University of Alberta

Concentration-dependent effects of long-term interleukin-1β treatment on spinal dorsal horn neurons in organotypic slice cultures

by

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Abstract

This thesis investigated the effects of interleukin-1β (IL-1β) on synaptic transmission of dorsal horn neurons in defined-medium organotypic slice cultures (DMOTC). IL-1β has been suggested to play a direct role in the pathophysiology of nerve injury and inflammation, and may be involved in the induction of neuropathic pain.

Experiments were carried out using infrared-differential interference contrast (IR-DIC) microscopy and whole-cell patch-clamp recordings from DMOTC neurons.

The main findings were that treatment with low concentration (1pM) IL-1β increased the amplitudes of spontaneous excitatory post-synaptic currents (sEPSCs) and decreased the frequency of spontaneous inhibitory post-synaptic currents (sIPSCs) in putative inhibitory neurons, while treatment with high concentration (100pM) IL-1β increased sEPSC amplitude and decreased sIPSC frequency in putative excitatory neurons. These data suggest that 1pM IL-1β changes the properties of putative inhibitory neurons to increase inhibitory transmission while 100pM IL-1β affects mainly putative excitatory neurons to increase excitatory transmission in the spinal dorsal horn.

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List of Abbreviations

aCSF – Artificial cerebrospinal fluid

ADP – Adenosine diphosphate

AM – Acetoxymethyl

AMP – Adenosine monophosphate

AMPA – α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

AP – Action potential

AP1 – Activator protein 1

AraC – Cytosine-β-D-arabino-furanoside

ATP – Adenosine triphosphate

BDNF – Brain-derived neurotrophic factor

BSA – Bovine serum albumin

BSS – Balanced salt solution

C_m – Cell capacitance

CAMP – Cathelicidin antimicrobial peptide; also known as LL37

CCI – Chronic constriction injury

CCL-2 – Chemokine (C-C motif) ligand 2; also known as MCP-1

CD4 – Cluster determinant 4

CD14 – Cluster determinant 14

CGRP – Calcitonin gene-related peptide

CNS – Central nervous system

COX-2 – Cyclo-oxygenase 2

CR-3 – Complement type-3 receptor

CRIDs – Cytokine-release inhibitory drugs

CVLM – Caudal ventrolateral medulla

CX3CL1 – Chemokine (C-X3-C motif) ligand 1; also known as fractalkine

CXCL8 – Chemokine (C-X-C motif) ligand 8; also known as IL-8

DMEM – Dulbecco's Modified Eagle Medium

DMOTC – Defined-medium organotypic slice culture

DNA – Deoxyribonucleic acid

DRG – Dorsal root ganglia

E-selectin – Endothelial-cell selectin

EGTA – (ethylenebis(oxyethylenenitrilo)) tetra-; also known as acetic acid

ELISA – Enzyme-linked immunosorbent assay

EPSC – Excitatory post-synaptic current

EPSP – Excitatory post-synaptic potential

GABA – γ-aminobutyric acid

GSTO-1 – Glutathione S-transferase-ω1-1

GTP – Guanosine triphosphate

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPA – Hypothalamic-pituitary-adrenal

ICAM1 – Intracellular adhesion molecule 1

icIL-1RA1 – Intracellular interleukin-1 receptor antagonist isoform 1

icIL-1RA2 – Intracellular interleukin-1 receptor antagonist isoform 2

icIL-1RA3 – Intracellular interleukin-1 receptor antagonist isoform 3

IL-1 – Interleukin-1

IL-1α – Interleukin-1α

IL-1β – Interleukin-1β

IL-1F5 – Interleukin-1 family, member 5

IL-1F6 – Interleukin-1 family, member 6

IL-1F7 – Interleukin-1 family, member 7

IL-1F8 – Interleukin-1 family, member 8

IL-1F9 – Interleukin-1 family, member 9

IL-1F10 – Interleukin-1 family, member 10

IL-1R1 – Type I interleukin-1 receptor

IL-1R2 – Type II interleukin-1 receptor

IL-1RA – Interleukin-1 receptor antagonist

IL-1RAcP – Interleukin-1 receptor accessory protein

IL-6 – Interleukin-6

IL-8 – Interleukin-8; also known as CXCL8

IL-10 – Interleukin-10

IL-18 – Interleukin-18

IL-18R – Interleukin-18 receptor

IPSC – Inhibitory post-synaptic current

IR – Infrared

IR-DIC – Infrared differential interference contrast

K-S test – Kolmogorov-Smirnov two-sample test

LPS – Lipopolysaccharide

LTP – Long-term potentiation

MAPK – Mitogen-activated protein kinase

MCP-1 – Monocyte chemoattractant protein-1; also known as CCL-2

MHC – Major histocompatibility complex

MMP – Matrix metalloproteinase

mRNA – Messenger ribonucleic acid

NF-κB – Nuclear factor-κB

NGF – Nerve growth factor

NMDA – N-methyl-D-aspartate

NO – Nitric oxide

NRM – Nucleus raphe magnus

NSAIDs – Nonsteroidal anti-inflammatory drugs

P2X7 – Purinergic receptor P2X, ligand-gated ion channel, 7

PAG – Periaqueductal gray matter

PBA – Parabrachial area

PBS – Phosphate-buffered saline

PGE_2 – Prostaglandin E_2

RMC – Nucleus reticularis magnocellularis

RMP – Resting membrane potential

RPGL – Nucleus reticularis paragigantocellularis lateralis

S100B – S100 calcium-binding protein; also known as neurite extension factor

SEM – Standard error of the mean

sEPSC – Spontaneous excitatory post-synaptic current

sIL-1R1 – Soluble type I interleukin-1 receptor

sIL-1R2 – Soluble type II interleukin-1 receptor

sIL-1RA – Secreted isoform of interleukin-1 receptor antagonist

sIL-1RAcP – Soluble interleukin-1 receptor accessory protein

sIPSC – Spontaneous inhibitory post-synaptic current

sPSC – Spontaneous post-synaptic current

SSRIs – Selective serotonin reuptake inhibitors

TIR – Toll-interleukin-1 receptor

TGF – Transforming growth factor

TLR – Toll-like receptor

TLR4 – Toll-like receptor 4

TNF – Tumor necrosis factor

TNF-α – Tumor necrosis factor-α

VGAT– Vesicular GABA transporter

VGLUT – Vesicular glutamate transporter

VPI – Ventral posterior inferior nucleus

VPL – Ventral posterior lateral nucleus

CHAPTER 1 GENERAL INTRODUCTION

1.1 Basics of Pain Transmission and Terminology

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Merskey & Bogduk, 1994). Nociception, the signaling of tissue damage which is often perceived as pain (Kandel & Kupfermann, 1995), arises from stimulation of a specific class of sensory neurons called nociceptors (Kunz et al., 2005). The free nerve endings of nociceptors transduce mechanical, thermal, or chemical tissue-damaging stimuli into electrical impulses (action potentials, or APs) that are transmitted along the axons of primary sensory neurons to the spinal cord or to homologous regions of the brain stem (Nestler, Hyman, & Malenka, 2001).

Nociceptive neurons, whose cell bodies are located in dorsal root ganglia, synapse in the dorsal horn of the spinal cord on local interneurons and on projection neurons (Nestler et al., 2001). From there, these projection neurons carry nociceptive information primarily to the brain stem, thalamus, and hypothalamus. These structures integrate the intensity, location, and context of the noxious stimulus and participate in the autonomic, alerting, and cognitive aspects of the pain response. Primary nociceptive neurons are classified according to responsiveness to stimuli, diameter of their axons, conduction velocity, and degree of myelination of their axons. Aδ-fibres are activated by intense mechanical stimuli or by noxious heat, have a diameter of 2-5μm, are thinly myelinated, and have a conduction velocity of 6-30 m/s (Burgess & Perl, 1967), while C-fibres are activated by dull yet noxious thermal, mechanical, and chemical stimuli, have a diameter of 0.2-1.5μm, lack myelination, and have a much slower conduction velocity of 0.5-2 m/s (Nestler et al., 2001; Perl, 1996).

1.1.1 Spinal Nociceptive Pathways

After nociceptive information has been processed and modulated in the dorsal horn, it is transmitted higher centres via several tracts originating from neurons located within the spinal cord grey matter (Willis, 1983; Brodal, 1992). The axons of these neurons travel within the spinal cord white matter and terminate in several regions, including the thalamus and brain stem nuclei (Mehler, Feferman, & Nauta, 1960). Nociceptive pathways in primates ascend from the anterolateral quadrant of the spinal cord to the brain, and the pathways that mediate pain include the spinothalamic tract (traditionally regarded as the most important), the spinoreticular tract, the spinomesencephalic tract, the spinocervical tract, the spinohypothalamic tract, the spinoparabrachial tract, the spinolimbic tract and the postsynaptic dorsal column pathway (Willis, 1983; Willis, 2006). Neurons in these pathways generally project contralaterally, and electrophysiological experiments suggest that the motivational-affective aspects are mediated by activity in spinothalamic tract neurons (Willis, 2006). Brain centres in the rat that receive major projections from the dorsal horn are the periaqueductal gray matter (PAG), parabrachial area (PBA), the caudal ventrolateral medulla (CVLM), and a number of thalamic nuclei such as the ventral posterior lateral nucleus (VPL), the ventral posterior inferior nucleus (VPI), the posterior complex in the lateral thalamus, and the central lateral nucleus in the medial thalamus; thus these ascending projections can modulate autonomic, endocrine, and cognitive functions (Spike, Puskar, Andrew, & Todd, 2003; Willis, 2006). There is evidence that there are plastic changes in the responses of spinothalamic tract neurons following capsaicin injection or peripheral neuropathy; these enhancements in transmission are similar to long-term potentiation (LTP) in certain brain structures and are a form of central sensitization (Willis, 2006).

In addition to activating brain systems involved in sensing and reacting to noxious stimuli, another role of the ascending nociceptive pathways likely includes engagement of descending control systems that will in turn modify pain transmission (Willis, 1983). The major descending pain pathways include multiple, pharmacologically distinct bulbospinal control systems, originating from projection neurons of the periaqueductal gray (PAG) and various subregions of the rostral ventral medulla, including the nucleus raphe magnus (NRM), the nucleus reticularis magnocellularis (RMC), and the nucleus reticularis paragigantocellularis lateralis (RPGL) (Basbaum & Fields, 1984). These terminate in the spinal dorsal horn, where their inhibitory action may be via direct postsynaptic inhibition, or via indirect mechanisms involving the release of opioid peptides, biogenic amines, and other transmitters (Basbaum & Fields, 1984). It is known that the spinoreticular tract participates in descending pain modulation (Basbaum & Fields, 1984). These control systems may not only be capable of responding to physiological pain transmission, but to cognitive and psychological events signaled by the brain as well, thus these systems may play a strong role in changing the nature of a potentially painful experience (Coren, Ward, & Enns, 2004).

1.1.2 Dorsal Horn Anatomy

The organization of the dorsal horn of the spinal cord was first described in the cat spinal cord by Rexed (1952). According to this model, the dorsal horn is composed of six laminae (laminae I-VI) that in turn consist of neurons of different sizes and densities (Nestler et al., 2001; Rexed, 1952). Primary nociceptive neurons synapse in the dorsal horn of the spinal cord on neurons in laminae I, II, IV, and V (see Figure 1-1) on both projection neurons and on local circuit interneurons (Nestler et al., 2001). These interneurons process afferent information and convey nociceptive information to motor neurons involved in local withdrawal reflexes and to autonomic neurons involved in spinal autonomic reflexes. They also modulate transmission to higher centres. Consequently, the dorsal horn is not simply a relay station but an important site of integration and modulation of both nociceptive and antinociceptive information (Nestler et al., 2001).

1.1.3 Superficial Dorsal Horn

The two most superficial layers of the dorsal horn, lamina I (also known as the marginal zone) and lamina II (also known as the substantia gelatinosa), receive Aδ- and C-fibre input and are particularly important in the processing of nociceptive information (Figure 1-2) (Snider & McMahon, 1998; Grudt & Perl, 2002). The majority (80%) of lamina I neurons are nociceptive, and a small proportion of these can project to a number of different brain nuclei via the spinothalamic, spinoreticular, and spinomesencephalic tracts (Craig & Dostrovsky, 1999; Todd, McGill, & Shehab, 2000). It is estimated that only 5% of lamina I neurons are projection neurons; the remaining nociceptive neurons are likely local excitatory and inhibitory interneurons (Dahlhaus, Ruscheweyh, & Sandkuhler, 2005; Spike et al., 2003). Of the lamina I neurons that are not nociceptors (20%) (Todd et al., 2000), most have been identified as either thermoreceptive neurons or wide dynamic range type neurons (Christensen & Perl, 1970). Lamina I neurons can be distinguished on the basis of morphology and intrinsic firing patterns; currently, three different morphological classes and four different firing patterns have been identified (Prescott & de Koninck, 2002).

A recent paper by Prescott & de Koninck (2002) reported that there is a significant correlation between morphological and physiological cell types, thus it is thought that there are three broad classes of neurons in lamina I (Figure 1-3). Tonic cells are neurons which are easily excited to fire action potentials and tend to fire regularly and throughout the duration of the depolarizing pulse; these tend to have fusiform morphology, that is, characterized by two primary dendrites emerging from opposite ends of the soma, elongated in the rostro-caudal axis. The second class, phasic cells, are able to achieve high firing rates in response to stronger stimuli, though they discontinue this firing abruptly before the end of stimulation; these tend to have pyramidal morphology, with three primary dendrites emerging longitudinally from separate points of a triangularly shaped soma. The last class comprised two different physiological types (delayed onset neurons and single spike neurons) with similar multipolar morphology, namely, having at least four primary dendrites with extensive arborization. Delayed onset neurons show a marked delay to the first action potential in response to a current pulse, while single spike neurons fire only a single action potential or a very short burst in response to the strong stimulation required to elicit any response from this cell type. The correlation of morphology with physiological (firing) properties has not been consistent across laboratories, indicating that differences in recording conditions, species, or age may play a role in proper characterization of lamina I neurons; thus, the categories defined above are still under investigation and should be interpreted with caution (Dougherty, Sawchuk, & Hochman, 2005).

While some lamina I neurons can project to a number of different regions in the CNS, it seems as though lamina II neurons are almost exclusively interneurons, whose axons synapse with projection neurons of lamina I or other interneurons in the substantia gelatinosa (Lu & Perl, 2005; Grudt & Perl, 2002). Lamina II neurons have been studied extensively and are known to integrate nociceptive input from afferent fibres via both excitatory and inhibitory interactions made possible by the intricate circuitry of the superficial dorsal horn (Melzack & Wall, 1965).

The morphology, electrophysiology, and connectivity of neurons in lamina II were recently characterized by Lu and Perl (2005), and based on this and other analyses, there are several main classes of neurons found in the substantia gelatinosa (Figure 1-4). Transient central neurons display a prompt but brief AP discharge in response to maintained step depolarization and have primarily rostrocaudal dendritic arborization. Vertical neurons have a delayed AP discharge in response to maintained step depolarization and display dorsoventral and rostrocaudal dendritic arborization. Lastly, islet neurons are characterized by a tonic AP discharge in response to maintained step depolarization and have extended, dense rostrocaudal dendritic arborization. A potential schema of the superficial dorsal horn connectivity is illustrated in Figure 1-5.

1.1.4 Synaptic Transmission in the Superficial Dorsal Horn

Glutamate is the predominant excitatory neurotransmitter in laminae I and II, and is released from Aδ and C-fibre nociceptors (Malcangio & Bowery, 1996). Projection neurons of lamina I and putative excitatory interneurons of lamina II are immunoreactive for the calcium binding protein, calbindin-D28K, which is thought to be a marker for excitatory (glutamatergic) neurons (Gamboa-Esteves, Kaye, McWilliam, Lima, & Batten, 2001; Polgar & Antal, 1995). In the rat lamina II, excitatory postsynaptic potentials (EPSPs) are caused by the release of glutamate from the primary afferents, which activates postsynaptic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), N-methyl-D-aspartate (NMDA), and kainate receptors (Yoshimura & Nishi, 1993). Kainate receptors can also directly regulate γ-aminobutyric acid (GABA) and glycine release in dorsal horn neurons by a presynaptic mechanism (Kerchner, Wang, Qiu, Huettner, & Zhuo, 2001).

GABA and glycine are the main inhibitory neurotransmitters in laminae I and II (Malcangio & Bowery, 1996). These neurotransmitters most likely act as cotransmitters (they are often released from the same vesicle, and both GABA and glycine receptors coexist at postsynaptic sites) and are thought to originate from local inhibitory interneurons (Malcangio & Bowery, 1996; Todd, 1996). Glycine immunoreactive neurons are present in laminae I and II, typically colocalized in cell bodies that are also GABA immunoreactive (Todd, 1996). Lastly, pharmacological blockade of spinal GABA receptors induces pain behaviours in rodents, suggesting that tonic GABAergic inhibition exists and maintains normal sensory responses (Malcangio & Bowery, 1996).

The complexity of the neuronal networks in the superficial laminae of the dorsal horn implies substantial processing and modulation of incoming activity; this can be accomplished by local interneurons, descending pro- or antinociceptive pathways, and chemical mediators released from neurons and glial cells (Grudt & Perl, 2002; Zeilhofer, 2005). Being the first site of synaptic integration of painful stimuli, the spinal dorsal horn is one of the regions that critically contributes to pathologically exaggerated pain sensations (Zeilhofer, 2005).

1.2 Neuropathic Pain

The ability of an animal to detect and react appropriately to an aversive stimulus is of fundamental importance to its survival (Clatworthy, 1999). Pain is the natural consequence of tissue injury and can serve as a biologically useful defense mechanism that warns against existing or imminent damage (Tsuda, Inoue, & Salter, 2005). In contrast to this nociceptive and acute pain, neuropathic pain (chronic, intractable pain due to nerve injury), serves no biological purpose and can be debilitating and cause extreme physical, psychological, and social distress (DeLeo & Colburn, 1999). This type of pain is no longer a protective signal of disease or injury, since it is chronic and outlasts the healing process; rather, the pain itself becomes the disease process.

Neuropathic pain typically develops after central or peripheral nerves are damaged, such as through trauma, surgery, cancer, diabetes, or infection (Loeser, 1990), and persists long after the initiating event has healed (Woolf & Salter, 2000). Neuropathic pain can also develop after nervous system dysfunction (Merskey & Bogduk, 1994), as in multiple sclerosis (Svendsen, Jensen, Hansen, & Bach, 2005) and amyotrophic lateral sclerosis (Galer et al., 2000). The prevalence of chronic pain in North America, Europe, and Australia in the general population is approximately 11% among adults (Harstall & Ospina, 2003), and neuropathic pain, a subset of chronic pain, is also common, with an estimated prevalence in the general population of 1.5% (Taylor, 2006). Neuropathic pain is among the most costly afflictions: current studies indicate that there is a significant use of health care resources by chronic pain sufferers (Harstall & Ospina, 2003), and in the U.S., annual healthcare charges are three-fold higher for neuropathic patients than for age- and sex-matched controls (Berger, Dukes, & Oster, 2004). Ironically, recent data suggests that these patients are not receiving the correct pharmacological therapy, and further, that almost one quarter are receiving no treatment for pain (Berger et al., 2004). In a study of 55 686 patients with painful peripheral neuropathies, the largest percentage of patients received a short-acting opioid for treatment (53.2%), and opioids of any type were the most commonly used class (53.9%) (Berger et al., 2004). This is cause for concern, since neuropathic pain is known to be poorly controlled by opioid analgesics (Watkins, Hutchinson, Johnston, & Maier, 2005; Arner & Meyerson, 1988): opioids provide partial relief at best, and are often used inappropriately and in excess (DeLeo & Colburn, 1999). The next largest percentage (39.7%) was being treated with nonsteroidal anti-inflammatory drugs (NSAIDs), which have no effect on neuropathic (i.e. non-inflammatory) pain (Argoff et al., 2006). Two other classes of agents with little or no evidence of efficacy in neuropathic pain, benzodiazepines and selective serotonin reuptake inhibitors (SSRIs), were also widely used, with 21.1% and 14.3% of patients, respectively, receiving them for treatment (Berger et al., 2004). Certain newer antiepileptic drugs, such as gabapentin and pregabalin, are currently the most effective drug therapy for some forms of neuropathic pain (Guay, 2005). The exact mechanism of action is not known, but both gabapentin and pregabalin bind to the $\alpha 2\delta$ subunit of voltage-gated calcium channels (N and P/Q) and may produce analgesia in inflammatory pain states by suppressing release of neuropeptides (e.g. substance P, calcitonin gene-related peptide [CGRP]) (Guay, 2005). Though these drugs have relatively widespread applicability (they are approved for the treatment of diabetic peripheral neuropathy and postherpetic neuralgia), both drugs require multiple daily dosing and can produce undesirable side effects (e.g. dizziness and/or somnolence; 21-26% incidence) (Guay, 2005). Currently, there is no universally effective treatment for neuropathic pain that parallels morphine for nociceptive pain (Woolf & Salter, 2000; Hansson & Dickenson, 2005; Zimmermann, 2001; Woolf, 2004).

Although neuropathic pain can arise from a variety of etiologically different clinical conditions, there are two nociceptive symptoms that are common to this disease: allodynia, which is pain due to a stimulus which does not normally provoke pain (e.g. clothing brushing against the skin being perceived as very painful instead of innocuous) and hyperalgesia, which is defined as an increased response to a stimulus which is normally painful (e.g. a pin prick on skin being perceived as very painful instead of slightly painful) (Merskey & Bogduk, 1994). Hyperalgesia is also a symptom of normal pain conditions that dissipates as tissue healing occurs; an example is the extreme pain sensation that is felt when one has a sunburn and takes a hot shower at a temperature that would normally (i.e. without a sunburn) cause slight discomfort. It must be emphasized that the mechanisms underlying these symptoms are not well understood, and that similar symptoms may not necessarily indicate similar mechanisms (Hansson & Dickenson, 2005).

The main thrust of research on neuropathic pain has been to investigate changes that occur in neurons and neuronal function in the peripheral and central nervous systems after trauma or inflammation (Tsuda et al., 2005). Molecular and cellular alterations are known to occur at various levels of the peripheral and central nervous systems after nerve injury and inflammation, and these result in neuronal plasticity and anatomical reorganization (Tsuda et al., 2005; Woolf & Salter, 2000; Zimmermann, 2001; Woolf, 2004). Although many of these changes are biologically useful and fade away once the initial insult subsides, some alterations can drive the pathological operation of the pain system if they become long-lasting and somewhat irreversible, such that the system is grossly and aberrantly modified (Woolf & Salter, 2000). The cellular processes that initiate and maintain neuropathic pain are poorly understood, though there are several mechanisms at various levels of the nervous system which have been suggested to be relevant (Hansson & Dickenson, 2005); these are: changes in neuronal behaviour and/or chemical environment leading to increased activity at and around the site of injury (Devor, Lomazov, & Matzner, 1994; Waxman, Dib Hajj, Cummins, & Black, 1999), changes in transmitter release through alterations in various channels (Luo et al., 2001; Matthews & Dickenson, 2001), spinal hyperexcitability, also called central sensitization (Dickenson, Matthews, & Suzuki, 2001), and increases in descending pain facilitation (Porreca, Ossipov, & Gebhart, 2002).

There is much literature to implicate central sensitization in the development of neuropathic pain (Woolf & Salter, 2000; Woolf, 1983; Dalal et al., 1999; Moore et al., 2002). Central sensitization arises when the heightened excitability of damaged axons spreads to higher centres, namely the dorsal horn of the spinal cord and brainstem nuclei (Sommer & Kress, 2004), and is characterized by spontaneous neuronal activity, lowered activation threshold, and increased response to a given stimulus in neurons of the dorsal horn of the spinal cord (Woolf & Salter, 2000; Woolf, 1983; Dalal et al., 1999; Moore et al., 2002). Modulation of the nociceptive pathway, specifically, disinhibition and spontaneous excitation of neurons in the dorsal horn, is a salient possible mechanism not only because it explains why pain persists after the initial stimulus subsides, but also because the cellular and molecular mechanisms producing this enhancement represent potential therapeutic targets.

To elucidate these cellular and molecular mechanisms, many recent studies have focused on the role of the immune system and glial cells in neuropathy and neuropathic pain (Tsuda et al., 2005; Watkins & Maier, 2003; Watkins, Milligan, & Maier, 2001a; Watkins, Milligan, & Maier, 2001b; McMahon, Cafferty, & Marchand, 2005; Watkins & Maier, 2004). It has been estimated that half of all clinical cases of neuropathic pain are associated with infection or inflammation of peripheral nerves rather than with nerve trauma (Said & Hontebeyrie-Joskowicz, 1992). Evidence has accumulated showing that injury to the nervous system is associated with activation and proliferation of glial cells (Tsuda et al., 2005; Stuesse, Cruce, Lovell, McBurney, & Crisp, 2000; Hashizume, DeLeo, Colburn, & Weinstein, 2000; Ohtori, Takahashi, Moriya, & Myers, 2004), and that stimuli that initiate neuropathic pain also initiate the activation of microglia within the CNS (Tsuda et al., 2005; Watkins, Maier, & Goehler, 1995; Watkins & Maier, 2002; Milligan et al., 2003). Moreover, general inhibitors of glial function attenuate neuropathy- or inflammation-induced hyperalgesia and allodynia (Milligan et al., 2003; Ledeboer et al., 2005; Meller, Dykstra, Grzybycki, Murphy, & Gebhart, 1994; Milligan et al., 2000; Sweitzer, Schubert, & DeLeo, 2001), further underscoring a significant role of glial cells in establishing neuropathic pain. As somewhat of a paradox, it must be remembered that although neuropathic pain is defined clinically as "non-inflammatory" pain, neuro-immune facilitatory cascades that occur at the site of injury and centrally are crucial in the establishment of central sensitization. Thus, although the traditional view is that chronic pain enhancement is mediated solely by neurons, accumulating evidence suggests that spinal cord glial cells importantly contribute to pain facilitation (Watkins & Maier, 2003; Watkins et al., 2001a; DeLeo & Yezierski, 2001; Ledeboer et al., 2005).

1.3 Role of Microglia in Neuropathic Pain

Neuronal and glial interactions mediate central nervous system (CNS) healing and repair as well as degeneration in response to nervous system injury (Coltman & Ide, 1996). Glial cells account for approximately 90% of the cells in the CNS and 50% of the volume in the brain and spinal cord (Vander, Sherman, & Luciano, 2001). Glial cells physically and metabolically support neurons, and can be divided into three groups: oligodendroglia, which form the myelin covering of CNS axons; astroglia , which metabolically sustain neurons, can stimulate neuronal growth, and may participate in neurotransmitter processing and information signaling in the brain; and microglia, which perform immune functions in the CNS and play a key role in pain hypersensitivity after nerve injury (Tsuda et al., 2005; Vander et al., 2001). The latter, when activated, are analogous to marchophages in the periphery; these two cell types share many phenotypic markers and effector molecules, making it difficult to distinguish between the two in pathological settings (Stoll & Jander, 1999).

Microglia represent 5-10% of glia in the CNS, and act as sensors for a range of stimuli that threaten physiological homeostasis, including CNS trauma, ischemia, and infection (Tsuda et al., 2005). Under normal conditions, microglia assume a resting, ramified form (Brierly & Brown, 1982), yet following nerve injury, inflammation, or chemical insult, microglia become activated and change their morphology to an ameboid, reactive form, morphologically and functionally similar to macrophages and monocytes (Tsuda et al., 2005; Coltman & Ide, 1996; Ledeboer et al., 2005; Piao et al., 2006).

Activation of microglia in the spinal cord dorsal horn, which is a relatively uniform process regardless of the underlying insult, also includes the ability to proliferate rapidly, as well as the upregulation of cell surface molecules such as complement type-3 receptor (CR-3), major histocompatibility complex (MHC) class I and II antigens, cluster determinants 4 and 14 (CD4, CD14), and toll-like receptor 4 (TLR4) (Stoll & Jander, 1999; Raghavendra, Tanga & DeLeo, 2003; Sweitzer, White, Dutta, & DeLeo, 2002; Tanga, Raghavendra, & DeLeo, 2004). Activated microglia synthesize and release a variety of potentially harmful soluble factors. These include reactive oxygen species, nitric oxide, peroxynitrite, proteolytic enzymes, arachidonic acid metabolites, and proinflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), all of which have the potential to contribute to central sensitization in the spinal dorsal horn (McMahon et al., 2005; Stoll & Jander, 1999).

In addition to the positive correlation between spinal microglial activation in vivo and the development or maintenance of pain hypersensitivity (Tsuda et al., 2005; Watkins & Maier, 2003), it has been shown that intrathecal injections of activated microglia simulate signs of neuropathic pain (Tsuda et al., 2003). Further, studies using fluorocitrate, a selective blocker of astrocyte and microglia metabolism (Hassel, Paulsen, Johnsen, & Fonnum, 1992), and the antibiotic minocycline, which inhibits microglial activation without affecting neurons or astrocytes (Zhang, Goetz, & Duncan, 2003), have shown that both compounds can block neuropathic states (Milligan et al., 2003; Ledeboer et al., 2005; Raghavendra et al., 2003). This evidence suggests that activated glia play a role in abnormal sensory processing, though the relative importance of microglia in various forms of pain is currently uncertain (McMahon et al., 2005), as are the precise mechanisms by which glia become activated (Tsuda et al., 2005). At present, there has not been any direct identification of factors that activate microglia, though many substances have been reported to trigger CNS microglia (McMahon et al., 2005), including ATP (Tsuda et al., 2003; Hide et al., 2000; Ferrari, Chiozzi, Falzoni, Hanau, & Di Virgilio, 1997), cytokines (TNF-α [Chao, Hu, & Peterson, 1995a; Chao, Hu, Sheng, & Peterson, 1995b; Chao, Hu, Sheng, Tsang, & Peterson, 1995c], IL-6 [Sheng, Hu, Kravitz, Peterson, & Chao, 1995], IL-1β [Chao et al., 1995a; Chao et al., 1995b; Chao et al., 1995c]), chemokines (CX3CL1, also known as fractalkine [Verge et al., 2004; Milligan et al., 2004], CCL-2, also known as MCP-1 [Tanaka, Minami, Nakagawa, & Satoh, 2004]), glutamate (Svensson, Hua, Protter, Powell, & Yaksh, 2003a), and peptides (substance P [Svensson et al., 2003b], CGRP [Priller, Haas, Reddington, & Kreutzberg, 1995], galanin [Priller, Hass, Reddington, & Kreutzberg, 1998]).

Many animal models of neuropathic pain, such as compression, ligation, or transection of the sciatic nerve (Bennett & Xie, 1988; Wall et al., 1979; Seltzer, Dubner, & Shir, 1990), are characterized by microglial activation post-injury, though the extent and time course of the changes can vary among the models (Colburn et al., 1997; Colburn, Rickman, & DeLeo, 1999). Spinal microglial activation in response to peripheral nerve injury can be observed within 24 hours, is maximal between days three and seven post-injury, and lasts for approximately four weeks (Tsuda et al., 2005; Coltman & Ide, 1996; Eriksson et al., 1993). There is no definitive marker for microglial cell activation (Guillemin & Brew, 2004), but many studies rely on immunocytochemistry for OX-42, which labels complement receptor type 3 (CR-3) (McMahon et al., 2005). Activated microglia exhibit upregulated OX-42 labeling (Coyle, 1998), and the temporal pattern of OX-42 upregulation in the dorsal horn correlates with the development of tactile allodynia and the development of pain hypersensitivity in a wide variety of nerve injury models (Watkins et al., 2001a; Stuesse et al., 2000; Colburn et al., 1999; Eriksson et al., 1993; Coyle, 1998). There is good evidence that one central mechanism in the manifestation of neuropathic pain is the release of pro-inflammatory cytokines (Watkins & Maier, 2003), and the ensuing upregulation of the enzyme cyclo-oxygenase 2 (COX-2) and subsequent release of prostanoids (McMahon et al., 2005). In particular, IL-1β is responsible, perhaps in conjunction with other cytokines such as IL-6 or IL-18, for central transcriptional activation of COX-2 after peripheral trauma and inflammation, and it is now known that COX-2 induction is the major limiting factor in the production or release of prostaglandins such as PGE_2 (Samad et al., 2001). There are a number of selective COX-2 inhibitors, such as celecoxib (Celebrex) (Penning et al., 1997) and rofecoxib (Vioxx) (Prasit et al., 1999) that are effective at treating pain due to inflammation (Clemett & Goa, 2000), but these have little efficacy in neuropathic pain conditions, most likely due to their poor CNS penetration. In point of fact, a recent study (Allan, Tyrrell, & Rothwell, 2005) using a more potent and more CNS penetrant COX-2 inhibitor, GW406381X, showed that this drug was effective in three models of neuropathic pain and central sensitization (rat chronic constriction injury [CCI], mouse partial ligation, and capsaicin-induced allodynia), further underscoring a major role for COX-2 induction in the onset of neuropathic pain following trauma-induced microglial activation.

To summarize, it is thought that immediately after nerve injury, damaged cells activate molecular pathways via a collection of chemical mediators that ultimately lead to microglial activation in the dorsal horn. Activated glia then release pro-inflammatory cytokines, among other chemical signals, and it is hypothesized that these cytokines, particularly IL-1β, initiate lasting changes in the properties of the spinal pain-processing network to bring about injury-induced pain hypersensitivity (Tsuda et al., 2005) (Figure 1-6).

1.4 The Interleukin-1 Family

The IL-1 family in mammals contains at least ten ligand proteins and 10 receptor molecules, and plays an important role in inflammation and host defense (DeLeo & Colburn, 1999; Allan et al., 2005; Huising, Stet, Savelkoul, & Verburg-van Kemenade, 2004). IL-1α and IL-1β were the first members of the IL-1 family to be identified (March et al., 1985). Although they are products of different genes, they have high sequence homology and exert similar biological effects, though IL-1β is more potent in activating humoral immune responses (Nakae, Asano, Horai, & Iwakura, 2001) and is predominant in the brain (Rothwell, 1991). They are synthesized as large precursor proteins by various cells of the peripheral and central immune system, such as lymphocytes and monocytes (Allan et al., 2005). Pro-IL-1α is biologically active and is cleaved by calpain to generate the smaller, mature protein; both of these forms remain mostly intracellular (Huising et al., 2004). Pro-IL-1β, on the other hand, is biologically inactive and requires cleavage by caspase-1 to produce the active 17kDa protein (Thornberry et al., 1992).

Both IL-1α and IL-1β bind the membrane-bound type I IL-1 receptor (IL-1R1), which then associates with the IL-1 receptor accessory protein (IL-1RAcP) to form a complex that allows intracellular signaling (DeLeo & Colburn, 1999; Sims et al., 1988; Korherr, Hofmeister, Wesche, & Falk, 1997). There is also a type II IL-1 receptor (IL-1R2), but it lacks an intracellular signaling domain, thus no downstream signal is initiated when IL-1 binds, and therefore, IL-1R2 is likely a decoy receptor (Subramaniam, Stansberg, & Cunningham, 2004). All three receptor molecules, IL-1R1, IL-1R2, and IL-1RAcP, can be shed from the cell membrane and consequently exist in soluble forms, sIL-1R1, sIL-1R2, sIL-1RAcP, respectively (Allan et al., 2005). sIL-1R2 and sIL-1RAcP both function as inhibitors of IL-1-mediated signal transduction by sequestering pro-IL-1β and IL-1R1, respectively. Additionally, it has also been proposed that signal transduction via IL-1R2 could be initiated if membrane-bound IL-1 were to bind the soluble IL-1R1 (sIL-1R1), and the subsequent association with IL-1RAcP occurred (Allan et al., 2005).

The third ligand member of the IL-1 family that was discovered is a naturally occurring competitive IL-1 receptor antagonist (IL-1RA) (Hannum et al., 1990). IL-1RA is produced by the same cells that express IL-1. There are three intracellular isoforms of IL-1RA: icIL-1RA1, icIL-1RA2, and icIL-1RA3, and there is one secreted isoform: sIL-1RA (Allan et al., 2005). The secreted isoform functions as a competitive antagonist that binds IL-1R1 but does not trigger signal transduction, while the other isoforms currently have poorly defined roles (Malyak, Smith, Abel, Hance, & Arend, 1998).

The fourth member of the IL-1 family to be discovered was IL-18 (formerly identified to be interferon-γ-inducing factor) (Allan et al., 2005). IL-18 is similar to IL-1β in that its pro-form is inactive and requires cleavage by caspase-1 to produce the mature, biologically active protein (Dinarello et al., 1998). IL-18 binds to the IL-18 receptor (IL-18R, previously known as IL-1-receptor-related protein), and both the IL-18 receptor and the IL-1 receptor belong to the IL-1 receptor and Toll-like receptor (TLR) superfamily, which plays a key role in the immune response (Dunne & O'Neill, 2003). These related receptors all have sequence similarity in their cytosolic regions: this conserved region has been termed the Toll-IL-1 receptor (TIR) domain, and it triggers a complex series of signaling events in the target cell that results in the activation of the transcription factor nuclear factor-κB (NF-κB) as well as the stress-related mitogen-activated protein kinases (MAPKs) (Dunne & O'Neill, 2003). This is followed by transcription of multiple inflammation-associated genes, including those that encode the following: chemokines (such as CXC-chemokine ligand 8 [CXCL8; also known as IL-8] and CX3C-chemokine ligand 1 [CX3CL1; also known as fractalkine], cytokines (such as IL-6 and tumor necrosis factor [TNF]), and adhesion molecules (such as endothelial-cell selectin [E-selectin] and intracellular adhesion molecule 1 [ICAM1]) (Subramaniam et al., 2004).

The remaining six putative ligand members of the IL-1 family are known as IL-1F5 (IL-1 family, member 5), IL-1F6, IL-1F7, IL-1F8, IL-1F9, and IL-1F10 (Allan et al., 2005). All members of this family are constitutively expressed in the brain at very low concentrations (Vitkovic, Bockaert, & Jacque, 2000). Since IL-1β is the most potent and well-characterized ligand agonist in the IL-1 family with respect to central nervous system function and injury, it will be the main focus of this section.

1.4.1 Regulation of IL-1β

A diverse range of stimuli can lead to changes in IL-1 expression at the mRNA and/or protein level (Allan et al., 2005). At the transcriptional level, gene expression of

IL-1β can be induced by various proinflammatory stimuli (such as bacterial and viral antigens), other cytokines (such as TNF), cellular injury, and hypoxia (Hsu & Wen, 2002; Perregaux, Bhavsar, Contillo, Shi, & Gabel, 2002). Transcription is also influenced by various factors that recognize and bind DNA regulatory sites, such as the cyclic AMP response element, the activator protein 1 (AP1) binding site, the NF-κB binding site, and the lipopolysaccharide (LPS)-response enhancer site (Watkins, Hansen, Nguyen, Lee, & Maier, 1999). Lastly, several other intracellular and extracellular signals can function to increase or decrease the rate of IL-1 transcription; factors that can increase transcription are complement components, prostaglandin E_2 (PGE_2), the β-chain of the S100 calcium-binding protein (S100B; also known as neurite extension factor), β-amyloid, and excitotoxins; factors that inhibit IL-1 transcription are glucocorticoids and their anti-inflammatory mediator, annexin-1 (also known as lipocortin-1) (Eriksson, Tehranian, Iverfeldt, Winblad, & Schultzberg, 2000; Liu, Li, Van Eldik, Griffin, & Barger, 2005; Relton et al., 1991). Regulation of IL-1 activity can also occur at the post-transcriptional and translational levels; for example, LPS can increase IL-1β protein production by increasing mRNA stability, translation can be increased by epidermal growth factor, corticotropin-releasing hormone, and ICAM1, while dexamethasone, a synthetic glucocorticoid, can inhibit IL-1 translation (Watkins et al., 1999; Kern, Warnock, & McCafferty, 1997).

At the post-translational level, the regulation of cellular release is also a control point for changes in IL-1 levels. The expression and cleavage of pro-IL-1β, and subsequent cellular release of IL-1β, may involve activation of P2X7 (a purinergic ligand-gated ion channel) by ATP in a caspase-1-dependent process that likely depends on the release of calcium from intracellular stores and on the activation of phospholipase C and phospholipase A_2 (Hazuda, Strickler, Kueppers, Simon, & Young, 1990; Perregaux & Gabel, 1994; Le Feuvre, Brough, Iwakura, Takeda, & Rothwell, 2002; Brough et al., 2003; Andrei et al., 2004). Treatment of microglial cells or macrophages with LPS induces expression of pro-IL-1β, but not cleavage or release. In LPS-primed macrophages, however, activation of P2X7 by ATP or ADP elicits both cleavage and release of IL-1β via caspase-1 (Le Feuvre et al., 2002). Another candidate molecule, the anti-microbial peptide LL37 (also known as CAMP) has been shown to be an inducer of IL-1β release through activation of the P2X7 receptor (Elssner, Duncan, Gavrilin, & Wewers, 2004). Lastly, a novel family of cytokine-release inhibitory drugs (CRIDs) have been shown to block IL-1β release independent of P2X7 activation (Perregaux et al., 2001); one of the main targets of CRIDs is glutathione S-transferase-ω1-1 (GSTO-1), an enzyme which may be a key regulator of IL-1β release (Laliberte et al., 2003).

1.4.2 IL-1β Involvement in CNS Injury and Inflammation

IL-1β that is produced during injury or inflammation to the central nervous system influences not only the local environment, but has actions that modify systemic functions, such as appetite, slow-wave sleep, body temperature, neuroendocrine function, and peripheral immune function (Allan et al., 2005; Vitkovic et al., 2000). These systemic responses, many of them contributing to host defense (i.e. sickness behaviour, fever, and the acute-phase response [Kelley et al., 2003]), are mediated by the effects of IL-1β on neuronal output and on hormonal influences, mainly through the release of corticotropin-releasing hormone and subsequent activation of the hypothalamic-pituitary-adrenal

(HPA) axis (Berkenbosch, van Oers, del Ray, Tilders, & Besedovsky, 1987). Conversely, the local effects of IL-1β on glial cells, neurons, endothelial cells, and immune cells that invade the brain during injury markedly contribute to local inflammation and may be involved in the induction and maintenance of neuropathic pain.

Interleukin-1β is secreted under pathological conditions associated with increased pain and hyperalgesia (Sommer & Kress, 2004; Watkins & Maier, 1999; Watkins et al., 1994; Davis & Perkins, 1994; Perkins & Kelly, 1994). Expression of IL-1β is upregulated rapidly (within hours) at the protein level following experimentally induced neurotoxic stimuli or animal models of neuropathic pain (Rotshenker, Aamar, & Barak, 1992; Allan et al., 2005). In particular, it has been shown that IL-1β production is enhanced in the periphery following crush injury to a peripheral nerve, and in microglia and astrocytes after CNS trauma (Rotshenker et al., 1992; DeLeo, Colburn, & Rickman, 1997; Sweitzer, Colburn, Rutkowski, & DeLeo, 1999). Although all endogenous brain cells (i.e. neurons, glial cells, and vascular endothelial cells) can express IL-1, early expression after injury is seen primarily in monocytes and macrophages, while later expression occurs in astrocytes (Boutin, Kimber, Rothwell, & Pinteaux, 2003). In addition to being associated with pain symptoms and conditions, IL-1β has been suggested to play a direct role in the pathophysiology of nerve injury and inflammation. Research has shown that IL-1β is capable of inducing neuropathic symptoms when delivered centrally or intrathecally (Watkins et al., 1994; Maier, Wiertelak, Martin, & Watkins, 1993; Ferreira, Lorenzetti, Bristow, & Poole, 1988; Fukuota, Kawatani, Hisamitsu, & Takeshige, 1994; DeLeo et al., 1997; Sweitzer et al., 1999; Oka, Oka, Hosoi, Aou, & Hori, 1995), though the most convincing evidence comes from studies using the IL-1 receptor antagonist or antibodies against IL-1β (Watkins et al., 1994; Maier et al., 1993; Safieh-Garabedian, Poole, Allchorne, Winter, & Woolf, 1995; Cunha, Cunha, Poole, & Ferreira, 2000; Sommer, Petrausch, Lindenlaub, & Tokya, 1999; Relton & Rothwell, 1992; Rothwell, 2003).

Injections or local administration of IL-1β induce mechanical and thermal hyperalgesia in the rat (Watkins et al., 1994, Maier et al., 1993; Ferreira et al., 1988), while IL-1β injected into the hind paw or knee joint has been shown to increase spontaneous firing of sensory nerves, increase neuronal responses to noxious heat and cold stimuli, and decrease the threshold for firing to pressure stimulation (Fukuota et al., 1994). Systemic administration of the IL-1 receptor antagonist, IL-1RA, has been shown to inhibit hyperalgesia mediated by lipopolysaccharide, lithium chloride, complete Freund's adjuvant, or IL-1 (Watkins et al., 1994, Maier et al., Safieh-Garabedian et al., 1995); while overexpression of endogenous IL-1RA markedly inhibits neuronal injury induced by ischaemia, excitotoxins, or trauma (Rothwell, 2003). Studies using antibodies also support the suggestion that IL-1β is neurotoxic and IL-1RA is neuroprotective: neutralizing antibodies to IL-1 receptors have been shown to reduce pain behaviours in mice with experimental neuropathy (Cunha et al., 2000; Sommer et al., 1999), antibodies that neutralize IL-1β or inhibit caspase-1 activity attenuate ischemic brain damage (Yamasaki et al., 1995; Hara et al., 1997), and administration of a neutralizing antibody specific for IL-1RA to rats undergoing occlusion of the middle cerebral artery induced a marked increase in the resultant neuronal injury (Loddick et al., 1997).

Although IL-1β administration has been shown to produce hyperalgesia in rats (Ferreira et al., 1988; Watkins et al., 1994), the nociceptive and anti-nociceptive effects of central IL-1β are still debated. There is evidence to suggest that IL-1β exerts biphasic responses on thermal and mechanical nociceptive thresholds depending upon the dosage: i.c.v. injection of IL-1β causes hyperalgesia at lower doses, but in contrast, can cause analgesia at higher doses (Coelho, Fioramonti, & Bueno, 2000; Oka, Aou, & Hori, 1993; Oka, Aou, & Hori, 1994; Rady & Fuigmoto, 2001). However, in general, studies have shown that exogenously injected IL-1β is pro-nociceptive in animals, and that blocking the actions of IL-1β can inhibit, prevent, or reduce these pro-nociceptive effects. Thus, there is good evidence for a role of IL-1β in the development and possibly the maintenance of central sensitization and neuropathic pain.

1.5 Organotypic Slice Culture

In vitro techniques are important for studying the physiological and pharmacological properties of neuronal circuits (Gahwiler, Capogna, Debanne, McKinney, & Thompson, 1997). Though acute slices of brain tissue are relatively easy to prepare and retain the cytoarchitechture of the original tissue, they are short-lived, and thus organotypic slice cultures were developed in an attempt to establish in vitro preparations that could be used for long-term studies (Gahwiler et al., 1997; Gahwiler, 1981). The defined-medium organotypic slice culture (DMOTC) technique used in this project is a modified roller tube technique for rat spinal cord slices (Lu et al., 2006), and was adapted from previously published methods (Gahwiler, 1981; Braschler, Iannone, Spenger, Streit, & Luscher, 1989; Ballerini & Galante, 1998). The value of the longevity of organotypic slice cultures in studying mechanisms of neuropathic pain and the effects of IL-1β is immense for two major reasons. First, the phenomenon of neuropathic pain is not quick to develop: many experiments have established that changes in pain transmission may arise from long-term changes in growth factors such as brain-derived neurotrophic factor (BDNF), inflammatory mediators such as prostaglandins, bradykinin, and cytokines, and neurotransmitters such as glutamate (McMahon et al., 2005; England, Bevan, & Docherty, 1996; Gold, Reichling, Schuster, & Levine, 1996). Secondly, production of IL-1β following injury or inflammation is known to occur over the course of one to two weeks (Coltman & Ide, 1996), thus, long-term applications of IL-1β are a prerequisite for studying its possible role in the development of neuropathic pain.

There are many factors to consider when using organotypic slice cultures in the place of acute slices or in vivo preparations. Age and development are of utmost concern, primarily because slice cultures are usually derived from early postnatal or embryonic animals while acute slices are taken from adult animals (Gahwiler et al., 1997; Howard, Walker, Michael, & Fitzgerald, 2005). With respect to age, organotypic cultures can be left to mature in vitro to allow for age-matched comparisons. Developmentally, although organotypic cultures are subject to differences in environmental cues, it has been recently shown that when matched for age, substantia gelatinosa neurons in DMOTC slices from embryonic rats are electrophysiologically and histologically similar to those in acute slices from young adult rats, suggesting that development in general proceeds similarly in both preparations (Lu et al., 2006). Another potential limitation of organotypic slice cultures is the use of serum-containing medium to maintain neurons (Gahwiler et al., 1997). Medium that contains serum contains undefined trophic components and cytokines, both of which can confound the effects of exogenously applied IL-1β; to minimize this, the culture system (DMOTC) used in this project is maintained with serum-free medium (Gahwiler et al., 1997; Lu et al., 2006).

1.5.1 Comparison of DMOTC Neurons With Neurons in Acute Slice Preparation

In a recent study, Lu et al. (2006) compared substantia gelatinosa neurons of DMOTC slices with those in age-matched acute slices from young adult rats, and found that these are electrophysiologically and histologically similar. In both preparations, neuronal morphology was found to be similar to previously defined sections of rodent dorsal horn (Grudt & Perl, 2002; Heinke, Ruscheweyh, Forsthuber, Wunderbaldinger, & Sandkuhler, 2004), and both displayed similarities in synaptic pharmacology and electrophysiological characteristics (Lu et al., 2006). Specifically, the five electrophysiological phenotypes that were delineated according to their action potential discharge patterns in response to a square pulse of depolarizing current from a holding potential of –60mV were similar to those seen in acute slices (Lu et al., 2006) (Figure 1-7). In DMOTC, "Tonic" neurons displayed a continuous, evenly spaced series of action potentials throughout the duration of the pulse (21.5% of neurons studied). "Irregular" neurons were characterized by an initial accommodation but subsequent action potential, and/or a rate of discharge that was disproportionate to the amount of injected current (27% of neurons studied). "Delay" neurons were defined as those that exhibited a clear delay before the initiation of action potential discharge (28.5% of neurons studied). "Transient" neurons discharged only a single action potential in response to all amounts of injected current (14% of neurons studied). Finally, "Phasic" neurons fired a short burst of action potentials and remained silent throughout the remainder of the current pulse (9% of neurons studied) (Lu et al., 2006). There was one main difference between neurons in DMOTC and those in acute slices: membrane excitability and spontaneous synaptic events were increased in the cultures (Lu et al., 2006), though this has been attributed to synaptic network properties of the cultures rather than to altered properties of individual neurons (Ballerini & Galante, 1998). The DMOTC culture described in this paper is identical to the one used in the current project.

1.6 Hypothesis

Currently, studies examining the effects of IL-1β on neuropathic pain are limited to acute behavioural and immunohistochemical experiments. Although these studies' findings form the basis of our current model of IL-1β function, they cannot further our understanding of the cellular mechanisms underlying the IL-1β-induced changes that occur after injury or inflammation of central or peripheral nerves. Further, there is evidence that microglia producing and releasing IL-1β continue to change in morphology for up to 10 days post-activation (Coltman & Ide, 1996), indicating that to mimic *in vivo* microglial activation and release of IL-1β, long-term applications of IL-1β are required. The defined-medium organotypic slice culture (DMOTC) technique developed in the Smith lab presents a unique and valuable opportunity to examine the long-term effects of IL-1β on neurons of the dorsal horn.

Using DMOTC, I have examined the concentration-dependence and time course of action of IL-1β on neurons of the dorsal region of the rat spinal cord. I have applied IL-1β for a seven-day period as this mimics the time course of microglial activation and spinal cytokine production in response to nerve injury (Coltman & Ide, 1996). My experiments provide much-needed insights into the long-term effects of IL-1β and the underlying cellular mechanisms.

My primary hypothesis is that long-term (7 days) application of IL-1β produces chronic changes in excitatory and inhibitory synaptic transmission. This was tested by employing two concentration paradigms, high (100pM) and low (1pM), as there is evidence that low, physiological doses and high, septic concentrations of IL-1β may have contrasting effects in the CNS (Desson & Ferguson, 2003). The experimental approach involved whole-cell patch-clamp recordings from dorsal horn neurons in defined-medium, organotypic slice culture (DMOTC) and 1-photon confocal imaging of cytosolic Ca^{2+} concentration.

Spinal dorsal horn connectivity

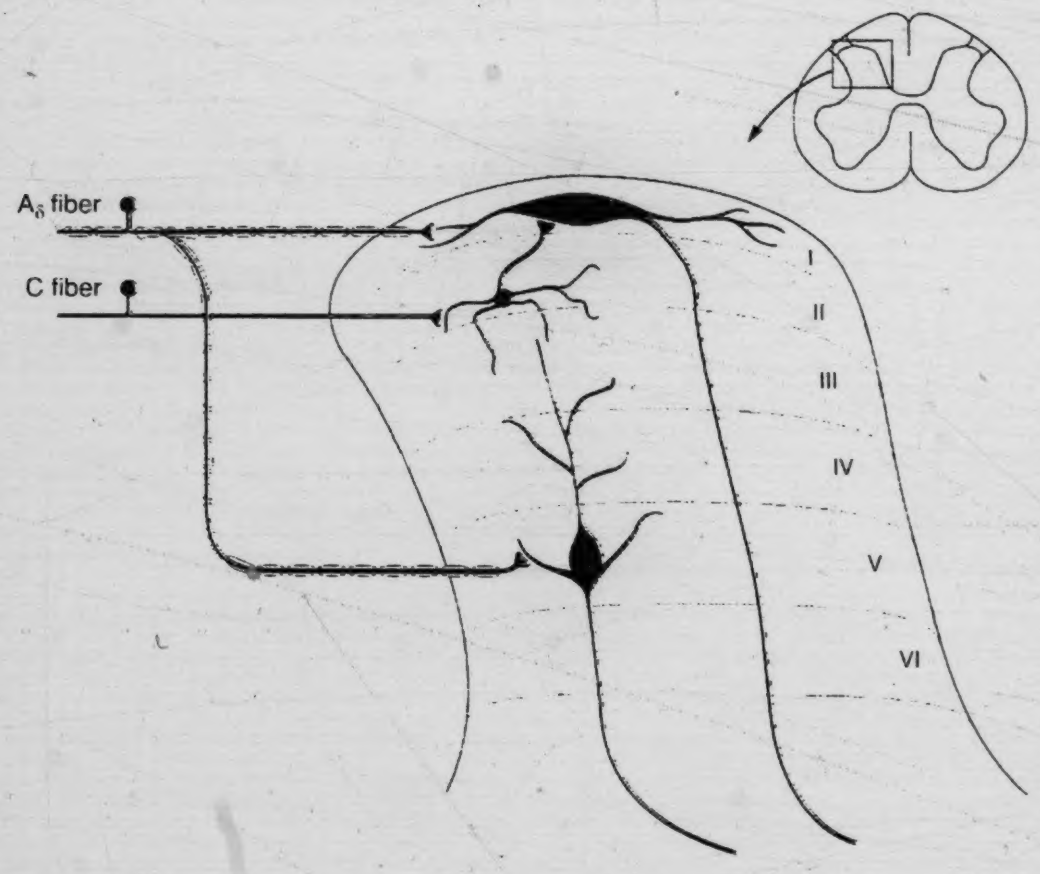

Figure 1-1. Laminae of the dorsal horn of the spinal cord with one of several possible synaptic arrangements of Aδ and C fibres. (Modified from Nestler et al., 2001).

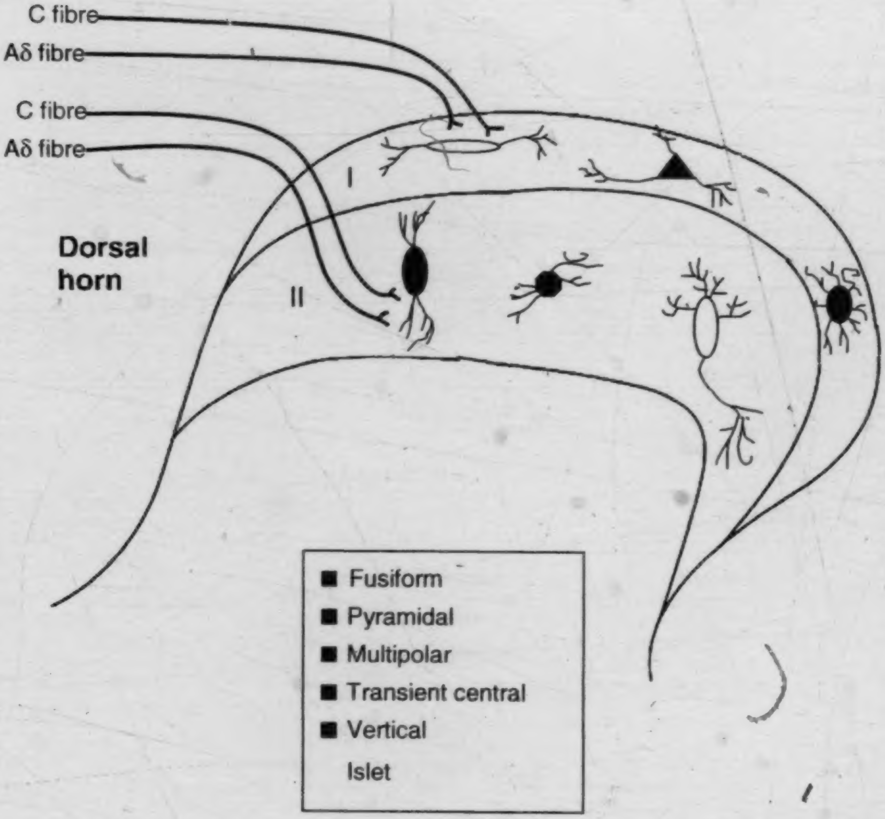

Figure 1-2. Schema of the innervation and cell types found in the superficial dorsal horn.

Lamina I neurons

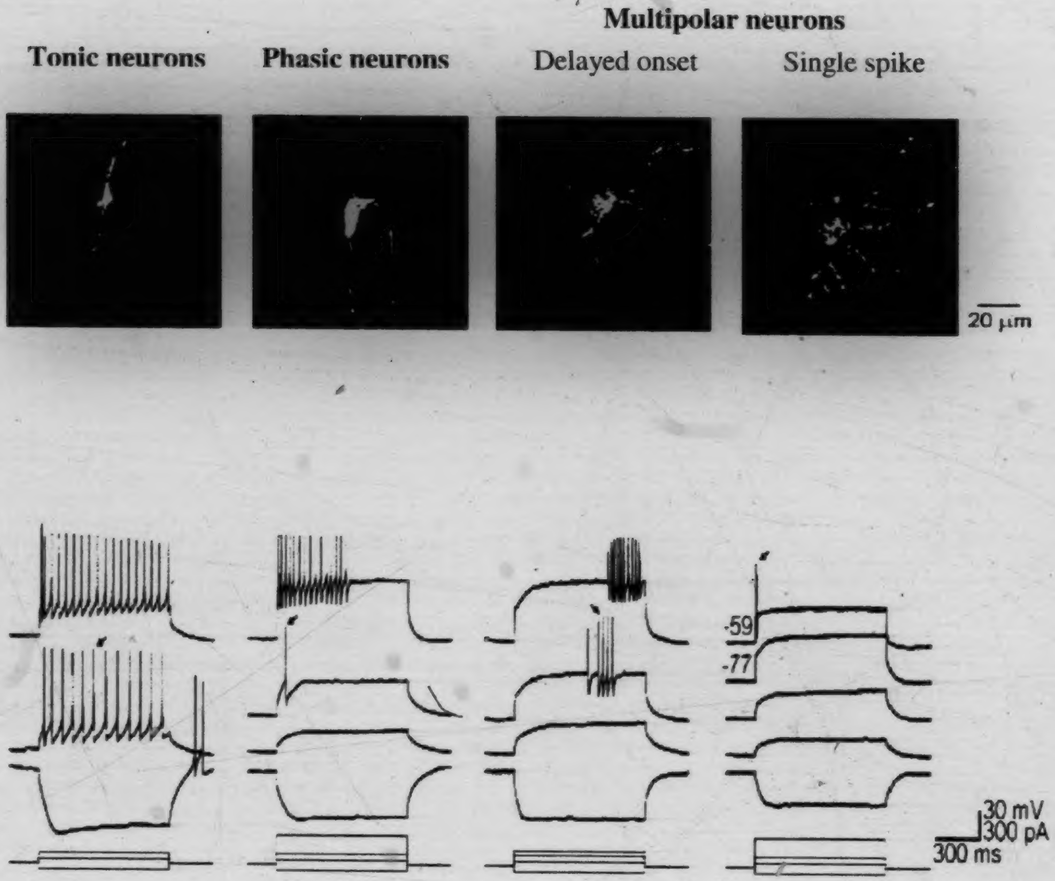

Figure 1-3. Tonic, phasic, and multipolar (delayed onset and single-spike) neurons of Lamina I. (Modified from Prescott & de Koninck, 2002).

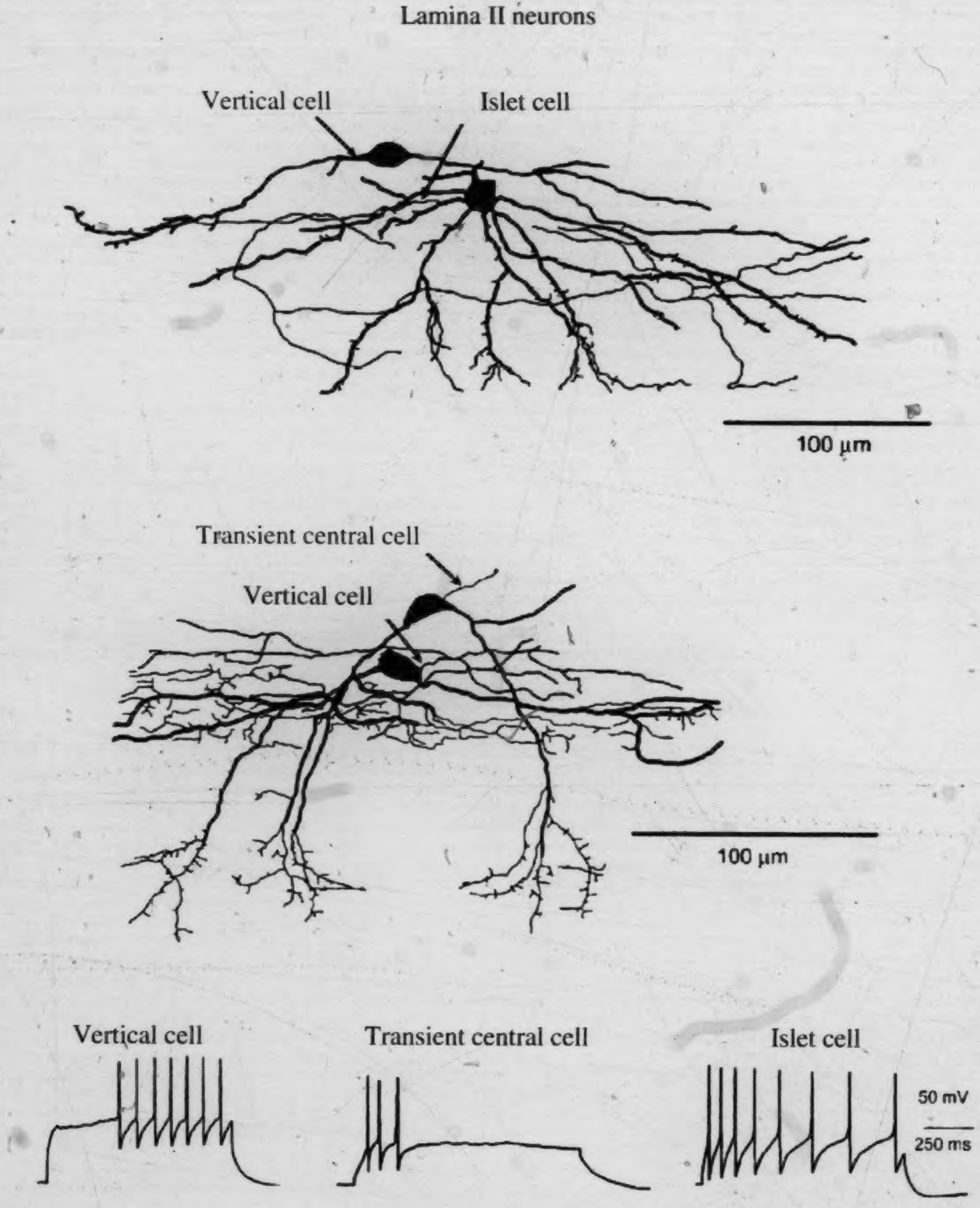

Figure 1-4. Transient central, vertical, and islet neurons of Lamina II. (Modified from Lu & Perl, 2005).

Possible superficial dorsal horn circuitry

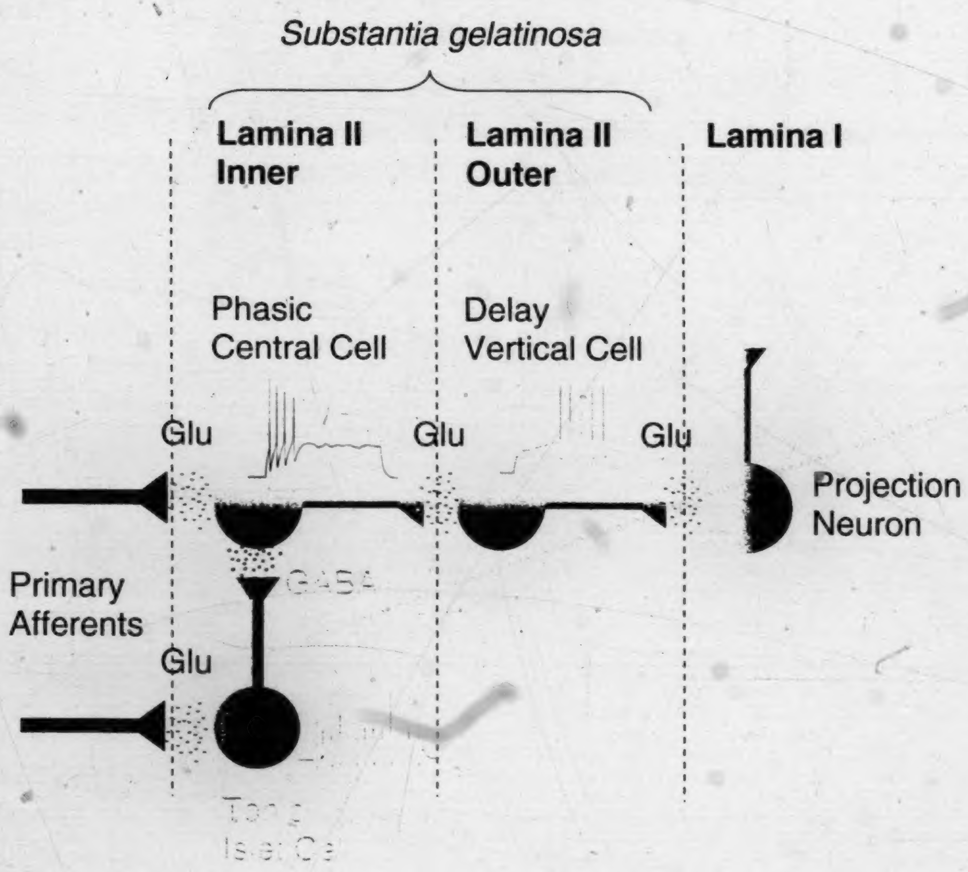

Figure 1-5. Schema of possible arrangement of synaptic circuitry in spinal superficial laminae of dorsal horn.

Microglial-mediated reactivity after nerve injury

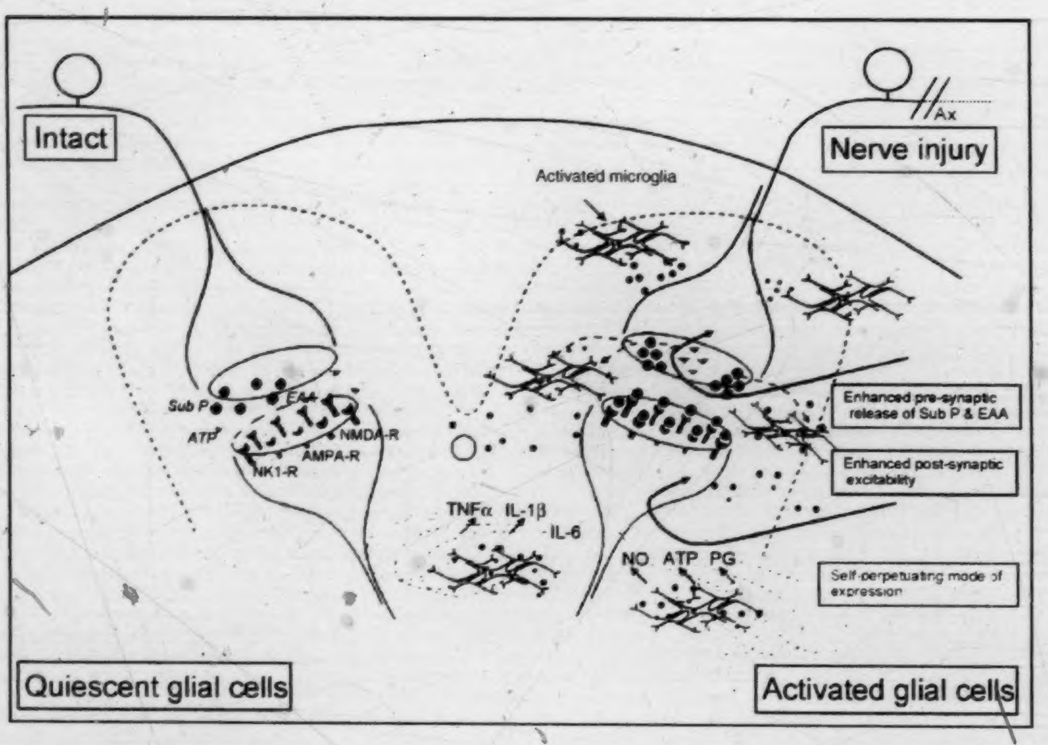

Figure 1-6. Summary of microglial activation and subsequent increase in reactivity following nerve injury or inflammation. (Modified from McMahon et al., 2005).

Electrophysiological phenotypes

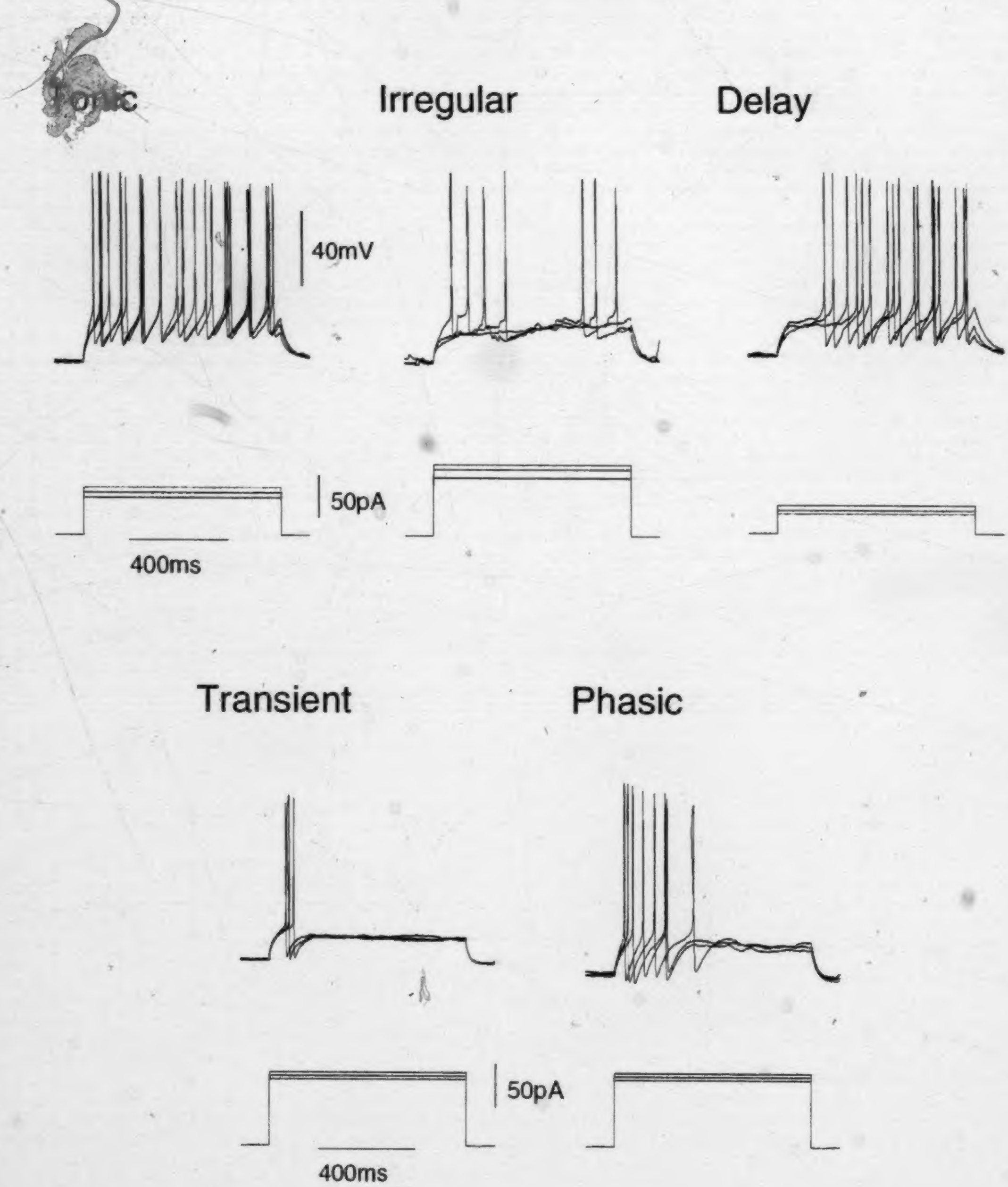

Figure 1-7. Electrophysiological phenotypes in DMOTC characterized by AP discharge patterns in response to a square pulse of depolarizing current. (Modified from Lu et al., 2006).

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CHAPTER 2 METHODOLOGY

All procedures were approved by the University of Alberta Health Sciences Laboratory Animal Policy and Welfare Committee, and thus comply with guidelines of the Canadian Council for Animal Care.

2.1 *Defined-Medium Organotypic Cultures*

The defined-medium organotypic slice culture (DMOTC) technique was adapted from previously published methods (Gahwiler, 1981; Braschler et al., 1989; Ballerini & Galante, 1998). Embryonic day 13-14 rat fetuses were delivered by caesarean section from timed-pregnant Sprague-Dawley rats (Charles River, Saint-Constant, PQ, Canada) under 2-5% isoflurane anaesthesia. Following the caesarean section, the female rat was euthanized with an overdose of intra-cardiac chloral hydrate (10.5%). Under aseptic conditions, the entire embryonic sac was placed in chilled Hanks' balanced salt solution (BSS) containing (in mM): 138 NaCl, 5.33 KCl, 0.44 KH_2PO_4, 0.5 $MgCl_2$-$6H_2O$, 0.41 $MgSO_4$-$7H_2O$, 4 $NaHCO_3$, 0.3 Na_2HPO_4, 5.6 D-glucose, and 1.26 $CaCl_2$. Individual rat fetuses were removed from their embryonic sacs and rapidly decapitated. Following this, the spine and dorsal tissue of each fetus was cut from the rest of the body and sliced into 275-325 μm transverse slices using a tissue chopper (McIlwain, St. Louis, MO, USA). Only lumbar spinal cord slices with an intact spinal cord, two attached dorsal root ganglia (DRGs) and associated muscle tissue were chosen and transferred to a new dish of Hanks' BSS. These slices were trimmed of excess ventral tissue and chilled for 1 hour at 4°C (Figure 2-1).

Embryonic spinal cord slices, with attached DRGs and some ventral muscle fibres, were plated onto clean glass coverslips (Karl Hecht, Sondheim, Germany) with a clot of reconstituted chicken plasma (lyophilized, 2mg/L heparin; Cocalico Biologicals Inc., Stevens, PA, USA) and thrombin (200 units/mL; Sigma, St. Louis, MO, USA). Coverslips were inserted into flat-bottomed tissue culture tubes (Nunc-Nalgene International, Mississauga, ON, Canada) filled with 1mL of culture medium, and placed into a roller drum (Model # TC-8, New Brunswick Scientific, Edison, NJ, USA) rotating at 120 rotations per hour in a dry heat incubator at 36°C (regular air-no CO_2; Figure 2-2). The initial medium in the tubes was composed of 82% Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, and 8% sterile water to adjust osmolarity (all from Invitrogen-Gibco, Grand Island, NY, USA). This medium was supplemented with 20ng/mL nerve growth factor (NGF) (Alomone Laboratories, Jerusalem, Israel) for the first 4 days. Antibiotic and antimycotic (5 units/mL penicillin G, 5 units/mL streptomycin, and 12.5 ng/mL amphotericin B; Invitrogen-Gibco) were also included in the medium during the first 4 days of culture. At the end of this 4 day period, the slices were treated with an anti-mitotic drug cocktail consisting of uridine, cytosine-β-D-arabino-furanoside (AraC), and 5-fluorodeoxyuridine (all from SIGMA, all at 10μM) for 24 hours to prevent the overgrowth of glial cells. Also during this treatment, the serum medium was halved with a serum-free, defined medium consisting of Neurobasal medium, 5mM Glutamax-1 and N2 supplement (all from Invitrogen-Gibco). Washout of the antimitotic drugs and serum medium was carried out on day 5 with the serum-free, defined medium described above. The medium within these tubes was exchanged with freshly prepared serum-free medium every 4-5 days, and DMOTC slices were maintained in serum-free, chemically-defined conditions until recordings were carried out.

2.2 Electrophysiology

For recording, slices were placed in a glass-bottomed chamber (25mm diameter, volume of ~1mL) and held in place with a circular platinum wire frame with attached parallel nylon fibres. Slices were superfused (flow rate ~ 1-2 mL/min) at room temperature (~22°C) with 95%O_2-5% CO_2 saturated artificial cerebrospinal fluid (aCSF) which contained (in mM): 127 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 25 D-glucose, pH 7.4 (320-325 mOsm).

DMOTC slices were viewed with a Zeiss Axioskop FS upright microscope equipped with infrared differential interference contrast (IR-DIC) optics and an IR sensitive video camera (NC-70, Dage-MTI, Michigan City, IN, USA). Cells for recording were chosen from within a light band located across the dorsal region of the slice. Whole-cell recordings were made from visually-identified dorsal horn neurons with an npi SEC 05L amplifier (npi Electronic, Tamm, Germany) in discontinuous single-electrode voltage-clamp or current-clamp mode. Switching frequencies were typically between 30-40 kHz. Signals were digitized at 10kHz and filtered at 0.5-2 kHz. Patch pipettes were pulled from thin-walled borosilicate glass (1.5 mm o.d., 1.12 mm i.d.; TW-150F-4, WPI, Sarasota, FL, USA). Pipettes for recording action potentials had resistances of 5-10 MΩ when filled with an internal solution containing (in mM): 130 D-gluconic acid, 2 $CaCl_2$, 10 HEPES, 10 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 0.2% Biocytin, adjusted to pH 7.2 with KOH (290-300 mOsm).

Spontaneous EPSCs and IPSCs (sEPSCs and sIPSCs) were digitized at 10kHz and filtered at 2kHz. Recording sEPSCs was done under voltage clamp at a holding potential of –70mV to minimize inhibitory currents. Recording sIPSCs was done under voltage clamp at a potential of 0mV to minimize excitatory currents. Voltage responses to current ramps were digitized at 10kHz and filtered at 1kHz. These recordings were done under current clamp at a holding potential of –60mV.

2.3 Interleukin-1beta treatment

IL-1β treatments were typically administered to DMOTC spinal cord slices 14 to 28 days after the start of culture, and recordings were typically obtained 21 to 35 days following the start of culture. IL-1β (Calbiochem, Hornby, ON, Canada) stocks, dissolved in bovine serum albumin (BSA; HyClone, Logan, UT, USA), were aliquoted into sterile vials in denominations of 100nM and 1nM; they were diluted using 0.1% BSA. Medium for the IL-1β-treated groups consisted of *n* mL of serum-free medium containing *n* μL of *x* nM IL-1β (x = 1 nM for 1 pM IL-1β group; x = 100 nM for 100 pM IL-1β group; *n* is the integer representing the volume of treatment media). To account for any unforeseen effects of BSA, control groups consisted of *n* mL of serum-free medium containing *n* μL of BSA. If 'day 1' is considered the treatment day, the DMOTC slices were exchanged on day 4 and recorded from on days 6-8 (Figure 2-3).

An enzyme-linked immunosorbent assay (ELISA) for IL-1β was conducted with our samples of BSA-treated control media, 1pM IL-1β-treated media, and 100pM IL-1β-treated media. As this was a precautionary test which was not part of the current project (i.e. ELISA was done by another laboratory), only one sample was measured after seven days' treatment of either BSA, 1pM, or 100pM IL-1β. As a corollary, the findings should be interpreted with caution as the reliability and validity of this ELISA have the potential to be low. Future analyses will need to be undertaken to definitively determine the concentrations of IL-1β after long-term treatment.

The reading from the control sample was below the detection limit for IL-1β, indicating that there is a negligible concentration of IL-1β in the BSA-treated control media. Following 1pM IL-1β treatment, it was found that the actual concentration in the media sample as detected by ELISA was 32.2pM. For the 100pM IL-1β treatment condition, it was found that the concentration in the media sample as detected by ELISA was 117.8pM. Not only are these values larger than expected based on the treatment concentrations, but they are not proportionately increased, suggesting that there may be different positive-feedback cascades or pro-inflammatory pathways activated with different concentrations. Additionally, the actual concentrations of each condition when presented on a logarithmic scale are not very different from one another: 32pM and 118pM (i.e. 1.5 and 2, since $\log 32 = 1.5$ and $\log 100 = 2$) are far closer than the intended 1pM and 100pM (i.e. 0 and 2, since $\log 1 = 0$ and $\log 100 = 2$), possibly weakening the attempt to obtain electrophysiological data from a "high" and a "low" concentration of IL-1β.

2.4 Histochemistry

Neurons in DMOTC were routinely filled with biocytin (0.2%) during recordings for post-hoc identification. This low concentration was chosen because biocytin has been reported to interfere with postsynaptic drug responses at higher concentrations (Eckert III, Willcockson, & Light, 2001). At the completion of recording, the patch pipette was slowly withdrawn from the cell and the slice transferred to cold (4°C) 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, and stored overnight at

4°C. Slices were rinsed three times with PBS and transferred to a 24-well tissue culture dish for staining. Slices were incubated with 0.3% Triton X-100 and streptavidin-Texas red conjugate (1:50 dilution, Molecular Probes, Eugene, OR, USA) for 50 minutes on a 3D rotator. Slices were thoroughly rinsed with distilled water, transferred to slides, allowed to dry overnight, and coverslipped with Cytoseal^{TM}-60 (Richard-Allan Scientific, Kalamazoo, MI, USA). A Zeiss LSM 510 confocal laser scanning microscope, equipped with an appropriate laser (HeNe1, wavelength 543 nm) and Texas red filter, was used to examine the tissue. Confocal images and 3D reconstructions were acquired using Zeiss LSM image browser software. Figures were converted to black and white to increase contrast and clarity.

2.5 Confocal Calcium Imaging

Voltage-activated Ca^{2+} channels are present throughout the nervous system, including in spinal dorsal horn neurons. Voltage-gated channels open or close, depending on the transmembrane potential of the cell. Depolarization of neurons occurs when a stimulus causes a critical number of voltage-gated Na^+ channels to open, allowing Na^+ ions to flow rapidly down their electrochemical gradient and into the cell. This change in voltage (depolarization) activates Ca^{2+} channels; accordingly, laser scanning confocal imaging of cytosolic Ca^{2+} concentration should be an appropriate tool to visualize neuronal activity and responsiveness to a high K^+ (depolarizing) pulse.

The membrane permeable acetoxymethyl (AM) ester of the Ca^{2+}-sensitive dye Fluo-4 (TEF Labs Inc. Austin, Texas, USA) was kept in 5 mM stock solution (a prediluted mixture of dimethyl sulfoxide and 20% pluronic acid; Invitrogen, Burlington, Ontario, Canada). Using aseptic techniques, 10μL of the Fluo-4 dye was added to the

DMOTC medium (1mL) of an individual flat-bottomed tissue culture tube, and incubated in the roller drum for 90 minutes. Fluorescence signals were measured using a confocal microscope and software (20x XLUMPlanF1-NA-0.95 objective; Olympus FV300, Carsen group, Markham, Ontario, Canada) as described previously (Nikolenko, Nemet, & Yuste, 2003). Fluorescence was sufficiently intense to reveal basic neuronal morphology and rhythmic changes in fluorescence intensity. The entire slice was fluorescently labeled, and individual cells were labeled at tissue depths up to 150 µm. Cellular activity and morphological features such as soma shape and array of primary dendrites could be resolved in tissue depths up to 60 µm. Neurons were typically imaged at a depth of 30-60µm. Up to 15 cells (typically 3-7) showed rhythmic Ca^{2+} rises in a single confocal x-y-image plane covering the Fluo-4-labelled spot. The spot was assessed using a 2-3x digital zoom at reduced settings for y-axis scanning. Consequently, such "clipped mode" imaging (instead of full frame acquisition of 512 x 512 pixels) was done from an area of about 300 x 100-220 µm. This provided scan rates of 0.7-0.8 seconds, sufficient to detect 70-100% of the peak Ca^{2+} rises. Morphological details of dorsal horn neurons were revealed by combining "z-stack"-imaging with 3D-animation using the Fluoview software. Since Fluo-4 fluoresces weakly in cells with low cytosolic Ca^{2+} concentrations, fluorescence was measured as slices were exposed to three different concentrations of high K^{+} external solution (20 mM, 35mM, and 50mM; normal external solution is 4mM) for 30 seconds.

2.6 *Statistical Analyses*

Mini Analysis Program software (Synaptosoft, Decatur, GA, USA) was used to distinguish sPSCs from the baseline noise and to prepare cumulative probability plots of amplitude histograms. The first 100 events after the first minute of recording spontaneous events were used in sPSC analysis. The Kolmogorov-Smirnov two-sample test (K-S test) was used to compare distributions of amplitudes and inter-event intervals in neurons in DMOTC. This method tested the null hypothesis that two independent samples come from populations that are identical with respect to location and distribution. The K-S test was used because it compared the entire distribution of amplitudes or inter-event intervals rather than the means of these data (Van der Kloot, 1991). Distributions were considered statistically significant if $p < 0.05$.

All data collected under voltage clamp and current clamp were acquired using pCLAMP 9.0 (Axon Instruments, Burlingame, CA,USA). Figures were produced with Origin 5.0 (OriginLab, Northampton, MA, USA). Statistical comparisons were made as appropriate using the unpaired t-test, Welsh corrected (GraphPad InStat software, San Diego, CA, USA), Tukey one-way ANOVA with post test (GraphPad InStat software, San Diego, CA, USA), or K-S test as described above. Statistical significance was taken as $p<0.05$ unless otherwise stated.

Embryonic spinal cord slice

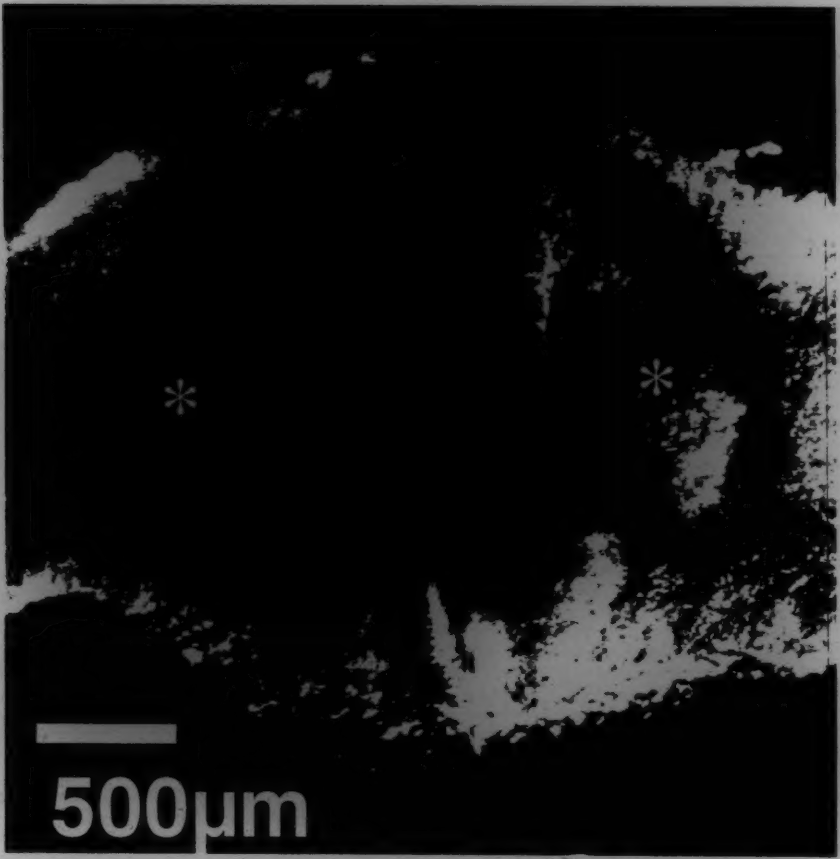
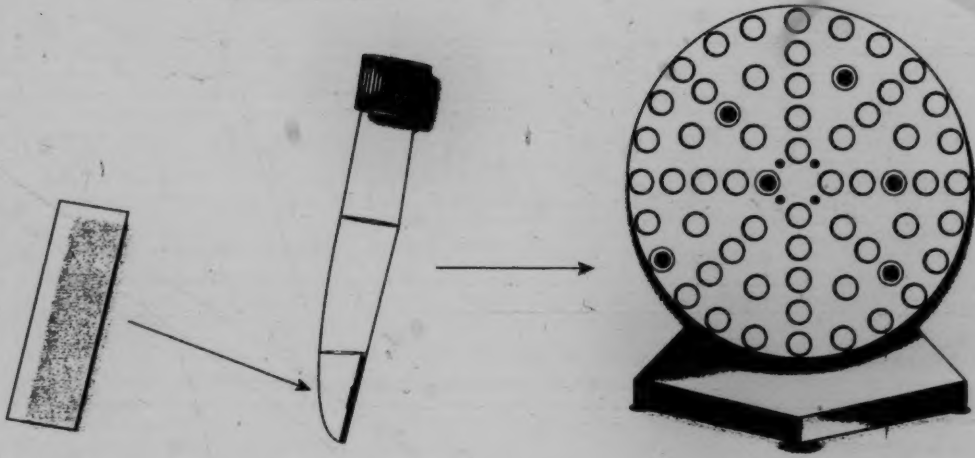


Figure 2-1. Spinal cord slice including DRGs (identified by asterisks) immediately before plating and incubation.



Flat-bottomed tubes and incubator

Figure 2-2. Diagram of incubation methods of flat-bottomed tubes with coverslips.

Treatment schedule

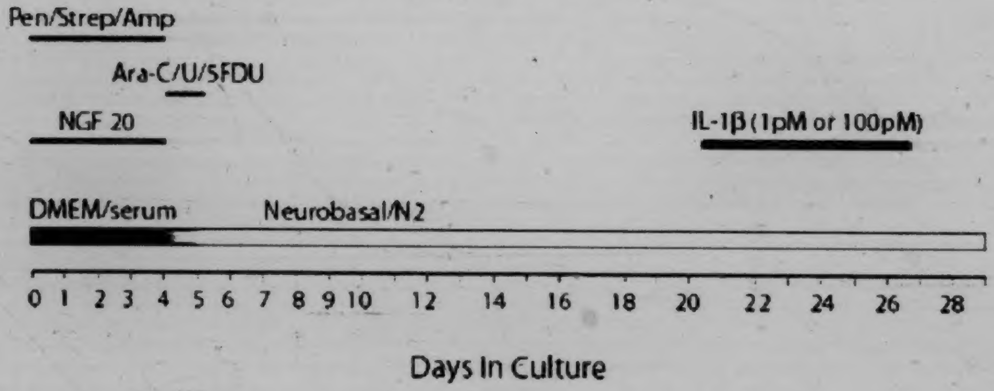


Figure 2-3. Time course of drug application and interleukin treatment in culture medium.

2.7 References

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Nikolenko, V., Nemet, B., & Yuste, R. (2003). A two-photon and second-harmonic microscope. *Methods, 30*: 3-15.

Van der Kloot, W. (1991). The regulation of quantal size. *Prog Neurobiol, 36*: 93-130.

CHAPTER 3 EFFECTS OF 100PM OF INTERLEUKIN-1BETA ON EXCITABILITY OF SPINAL DORSAL HORN NEURONS IN DMOTC

3.1 Effects of a High Concentration (100pM) of IL-1β

In the DMOTC, there were five different neuronal classes that were characterized according to electrophysiological properties, as was discussed in Chapter 1 and as has been previously described (Lu et al., 2006). The relative proportions of each cell type to the entire population of neurons was found for BSA controls, as well as for the IL-β-treated conditions (Figure 3-1).

In controls, it was found that 22.9% of cells were Tonic, 47.9% were Irregular, 10.4% were Delay, 10.4% were Transient, and 8.33% were Phasic. When examining the cells treated with 100pM of IL-1β, it was found that 17.1% were Tonic, 40% were Irregular, 22.9% were Delay, 11.4% were Transient, and 8.57% were Phasic. These proportions are relatively similar to each other, and to the proportions which have been previously reported in DMOTC cultures (Lu et al., 2006).

3.1.1 Resting Membrane Potential, Rheobase, Cell Capacitance

In BSA-treated control cells, there were some differences between the five different neuronal classes with respect to resting membrane potential (RMP), rheobase, input resistance, and cell capacitance (C_m); these are summarized on Table 3-1. Average RMPs ranged from –45mV to –56mV, average rheobase ranged from 25pA to 135pA, and average C_m ranged from 30pF to 57pF. The main trends among the neuronal types were that Tonic cells had the lowest rheobase of all the groups (average = 25 pA), while Transient neurons had the highest rheobase (average = 135 pA) and had the smallest average C_m (average = 30.333 pF). The only statistically significant difference was that rheobase was significantly lower in Tonic neurons when compared to Transient neurons (p=0.0082).

The RMP, rheobase, and C_m of the five neuronal classes in the high concentration (100pM) of IL-1β condition were not significantly different than the corresponding neuronal types in the control group (p values ranged from 0.12 to 0.99; Figures 3-2, 3-3, and 3-4). Average RMPs ranged from –44mV to –54mV, average rheobase ranged from 32pA to 174pA, and average C_m ranged from 23pF to 48pF. Also, similar trends were observed: Tonic cells had the lowest average rheobase of all the neuronal types (31.667 pA), while Transient cells had the highest average rheobase (173.75 pA) and the smallest average C_m (23 pF). Statistically significant differences within the 100pM group were that the rheobase of Transient neurons was higher than Tonic, Delay, and Irregular neurons ($p<0.05$).

3.1.2 Spontaneous Post-Synaptic Currents

The amplitudes and frequencies of excitatory and inhibitory spontaneous post-synaptic currents (sEPSCs and sIPSCs, respectively) were measured in both the BSA-treated control cells and the IL-1β-treated cells. The significant differences between sPSCs of IL-1β-treated cells and controls are summarized in Table 3-2.

With respect to sEPSCs, it was found that amplitudes were significantly greater following IL-1β treatment in Tonic cells, Delay cells, and Transient cells (Figures 3-5, 3-7, and 3-11). In contrast, amplitudes were significantly reduced following IL-1β treatment in Irregular cells and Phasic cells (Figures 3-9 and 3-13). sEPSC frequencies were significantly reduced following IL-1β treatment in Delay cells, Irregular cells, and Phasic cells, but significantly greater following IL-1β treatment in Transient cells; lastly, in Tonic cells, there was no significant difference in sEPSC frequency (Figures 3-5, 3-7, 3-9, 3-11, and 3-13).

In terms of sIPSCs, it was found that amplitudes were significantly greater following IL-1β treatment in Irregular cells, Delay cells, and Transient cells, while in Tonic cells and Phasic cells, amplitudes were significantly reduced (Figures 3-6, 3-8, 3-10, 3-12, and 3-14). sIPSC frequencies were significantly reduced following IL-1β treatment in Tonic cells, Irregular cells, and Delay cells, but significantly greater in Transient cells, and not significantly different than controls in Phasic cells (Figures 3-6, 3-8, 3-10, 3-12, and 3-14).

Changes in sPSC amplitude and frequency were quite diverse across cell types and treatment conditions. Both increases and decreases in sIPSC and sEPSC amplitude were seen, but there was a general trend towards a decreased frequency of sEPSCs and sIPSCs in all cell types, with the exception of Transient neurons. The number of cells in the Phasic and Transient groups were low, which is a rationale for excluding these from further analysis.

Table 3-1

Resting Membrane Potential, Rheobase, and Cell Capacitance Means By Neuronal Phenotype and Treatment Condition

BSA Controls

	Tonic	**Irregular**	**Delay**	**Transient**	**Phasic**
RMP (mV)	*x* = −52.455 SE = 2.626 *n* = 11	*x* = −51.261 SE = 1.775 *n* = 23	*x* = −48.600 SE = 5.913 *n* = 5	*x* = −45.000 SE = 5.244 *n* = 5	*x* = −56.250 SE = 4.090 *n* = 4
Rheobase (pA)	*x* = 25.000 SE = 2.041 *n* = 9	*x* = 82.500 SE = 12.101 *n* = 18	*x* = 61.000 SE = 19.962 *n* = 5	*x* = 135.000 SE = 32.851 *n* = 4	*x* = 118.750 SE = 53.205 *n* = 4
Capacitance (pF)	*x* = 51.000 SE = 8.631 *n* = 9	*x* = 48.125 SE = 6.347 *n* = 16	*x* = 38.800 SE = 3.353 *n* = 5	*x* = 30.333 SE = 9.871 *n* = 3	*x* = 57.000 SE = 22.855 *n* = 3

100pM IL-1β

	Tonic	**Irregular**	**Delay**	**Transient**	**Phasic**
RMP (mV)	*x* = −53.167 SE = 4.556 *n* = 6	*x* = −53.643 SE = 1.880 *n* = 14	*x* = −54.625 SE = 3.664 *n* = 8	*x* = −45.000 SE = 5.401 *n* = 4	*x* = −44.000 SE = 4.583 *n* = 3
Rheobase (pA)	*x* = 31.667 SE = 10.138 *n* = 6	*x* = 70.833 SE = 11.947 *n* = 12	*x* = 72.143 SE = 8.441 *n* = 7	*x* = 173.750 SE = 65.903 *n* = 4	*x* = 105.000 SE = 20.000 *n* = 2
Capacitance (pF)	*x* = 31.400 SE = 7.711 *n* = 5	*x* = 40.333 SE = 3.793 *n* = 12	*x* = 48.286 SE = 7.318 *n* = 7	*x* = 23.000 SE = 3.782 *n* = 5	*x* = 28.500 SE = 3.500 *n* = 2

1pM IL-1β

	Tonic	**Irregular**	**Delay**	**Transient**	**Phasic**
RMP (mV)	*x* = −57.857 SE = 3.776 *n* = 7	*x* = −48.857 SE = 2.132 *n* = 7	*x* = −50.556 SE = 2.212 *n* = 9	*x* = −70.000 SE = n/a *n* = 1	*x* = −65.000 SE = n/a *n* = 1
Rheobase (pA)	*x* = 33.333 SE = 6.146 *n* = 6	*x* = 45.000 SE = 9.354 *n* = 5	*x* = 40.000 SE = 12.649 *n* = 5	*x* = n/a SE = n/a *n* = n/a	*x* = 65.000 SE = 5.000 *n* = 2
Capacitance (pF)	*x* = 47.833 SE = 8.031 *n* = 6	*x* = 34.800 SE = 6.793 *n* = 5	*x* = 40.750 SE = 7.804 *n* = 4	*x* = n/a SE = n/a *n* = n/a	*x* = 35.000 SE = 10.000 *n* =2

Table 3-2

Summary of Significant Differences in Spontaneous Post-Synaptic Currents (sPSCs) of IL-1β-Treated Cells When Compared With BSA Controls (Statistical Significance Determined with K-S Test, $p<0.05$)

	TONIC		IRREGULAR		DELAY		TRANSIENT		PHASIC	
1pM IL-β sEPSCs	Amplitude ↑	Frequency NS p=0.10	Amplitude ↓	Frequency ↑	Amplitude ↑	Frequency ↓	Amplitude ----	Frequency ----	Amplitude NS p=0.07	Frequency ↓
1pM IL-β sIPSCs	Amplitude ↑	Frequency ↓	Amplitude ↓	Frequency ↑	Amplitude ↑	Frequency ↓	Amplitude ----	Frequency ----	Amplitude ↑	Frequency ↓
100pM IL-β sEPSCs	Amplitude ↑	Frequency NS p=0.40	Amplitude ↓	Frequency ↓	Amplitude ↑	Frequency ↓	Amplitude ↑	Frequency ↑	Amplitude ↓	Frequency ↓
100pM IL-β sIPSCs	Amplitude ↓	Frequency ↓	Amplitude ↑	Frequency ↓	Amplitude ↑	Frequency ↓	Amplitude ↑	Frequency ↑	Amplitude ↓	Frequency NS p=0.37

Neuronal type proportions

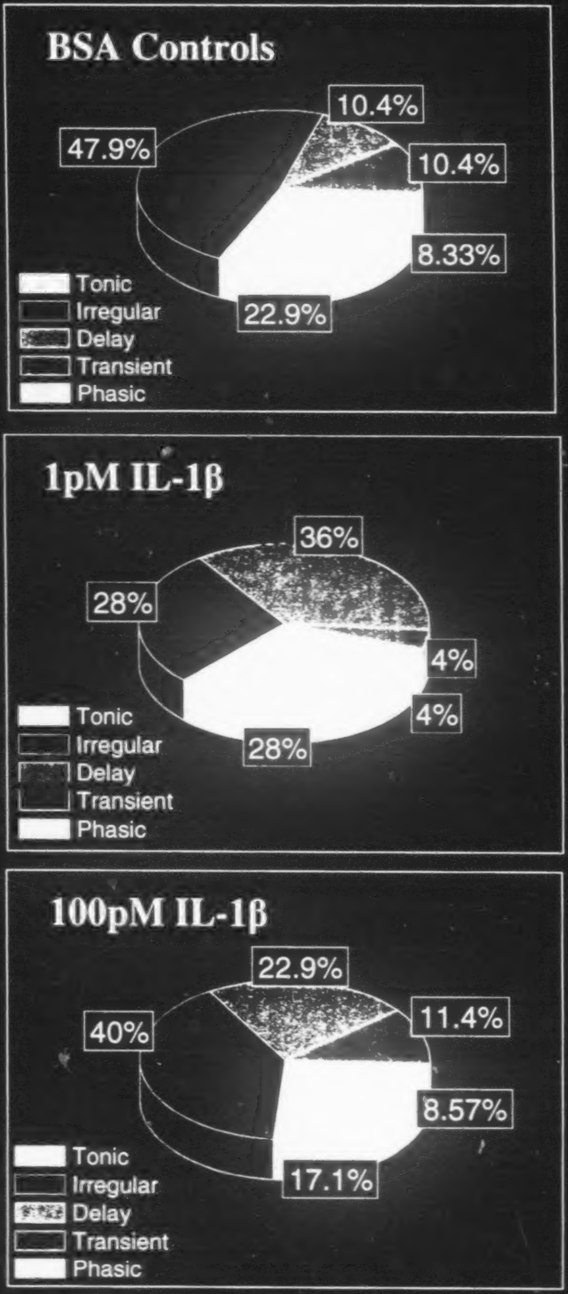


Figure 3-1. Proportions of each neuronal type (by treatment condition)found in the dorsal region of DMOTC slices.

RMP – BSA controls and 100pM IL-1β

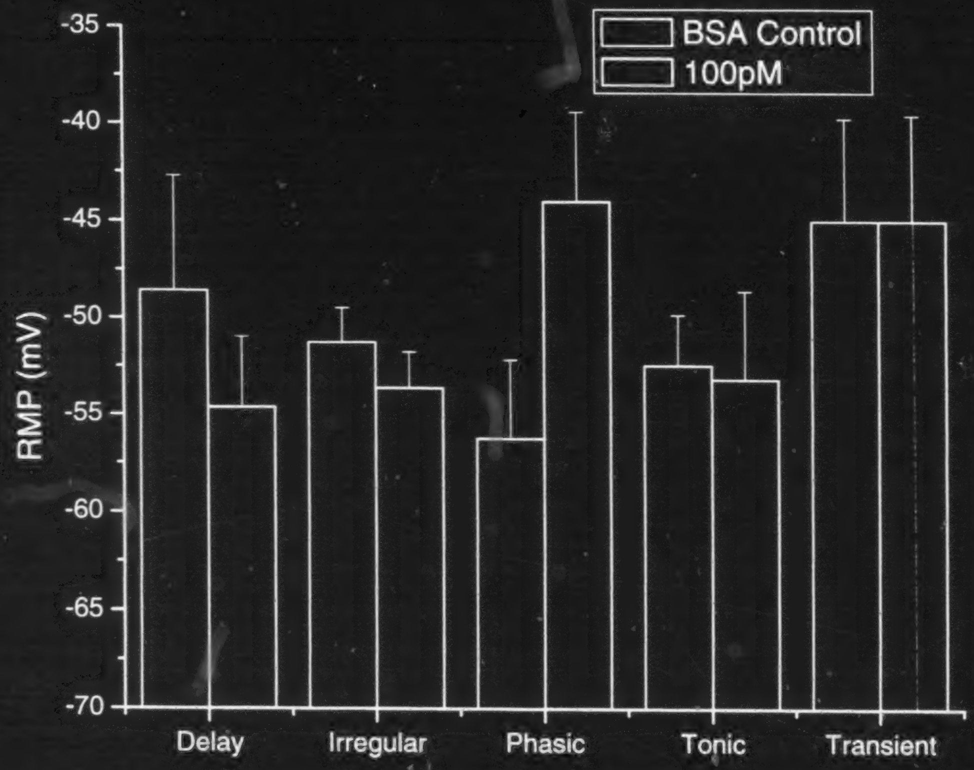


Figure 3-2. Comparison of resting membrane potential of BSA control neurons and neurons treated with 100pM IL-1β. (Error bars = SEM in this and all subsequent figures; for sample sizes '*n*', refer to Table 3-1).

Rheobase – BSA controls and 100pM IL-1β

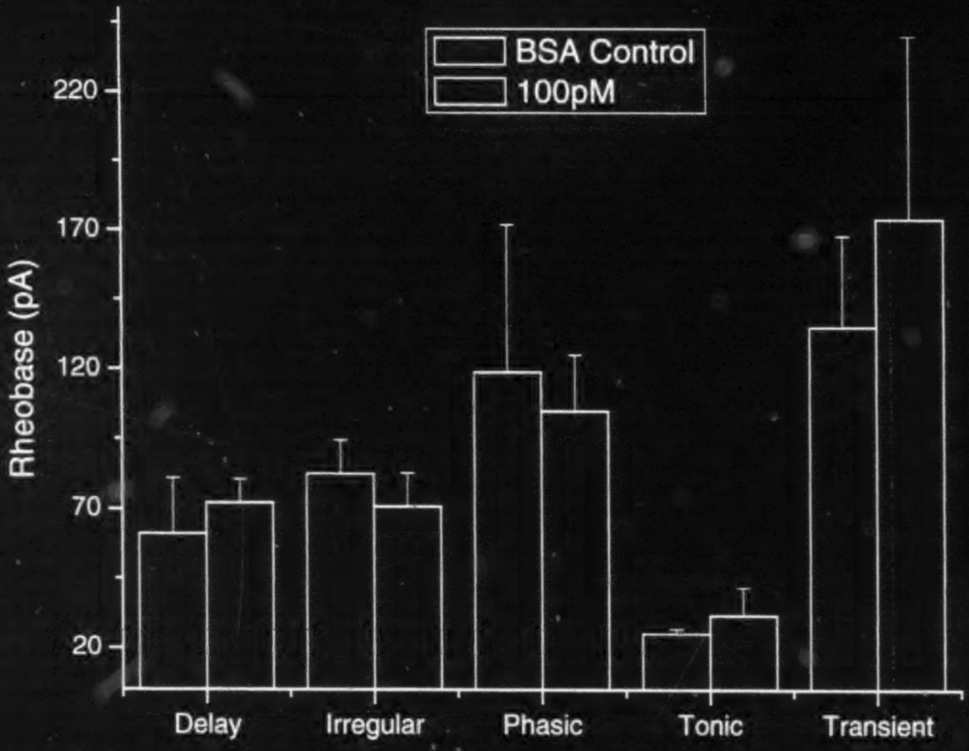


Figure 3-3. Comparison of rheobase of BSA control neurons and neurons treated with 100pM IL-1β.

C_m – BSA controls and 100pM IL-1β

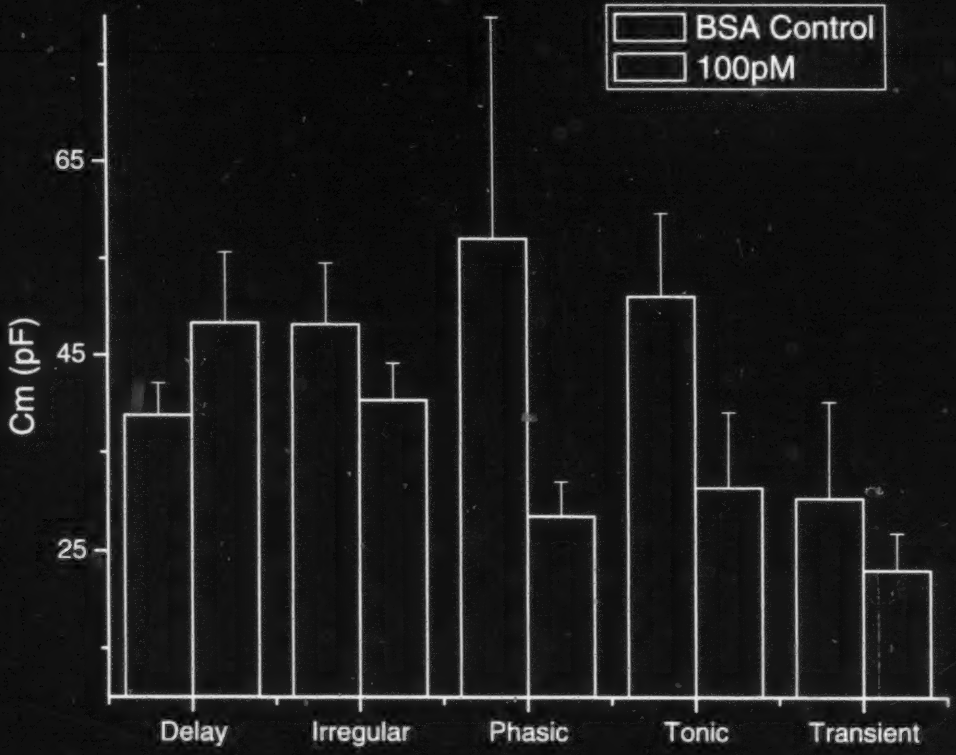


Figure 3-4. Comparison of cell capacitance of BSA control neurons and neurons treated with 100pM IL-1β.

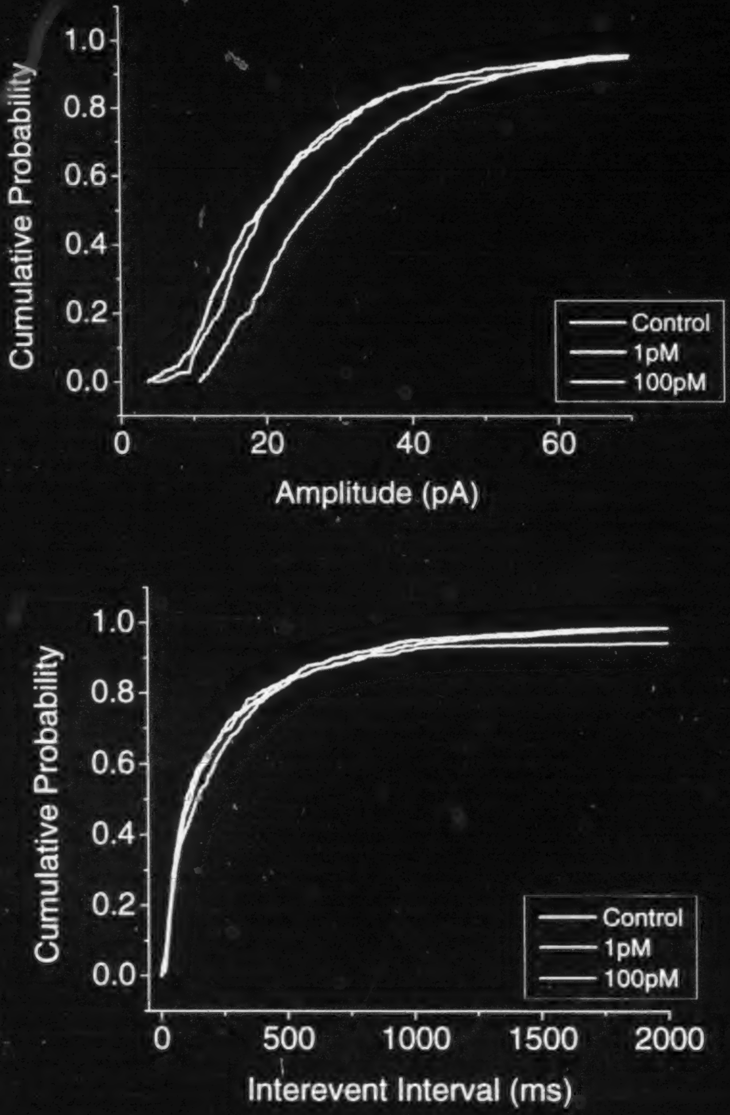

Tonic sEPSCs

Figure 3-5. Cumulative probability distribution of sEPSC amplitudes and interevent intervals in Tonic neurons treated with BSA or IL-1β. Cumulative probability plots from this point onwards represent the first 100 events after the first minute of recording and comprise between two and 16 cells (see Table 3-1).

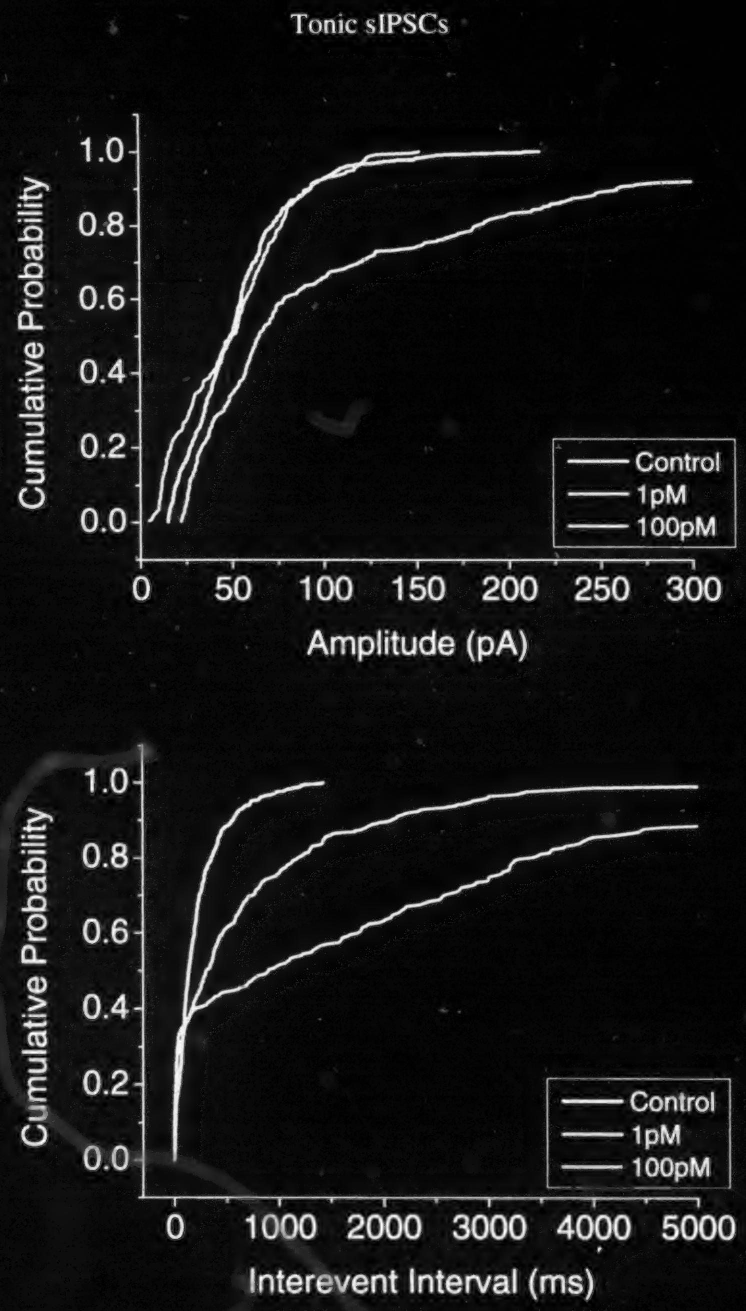


Figure 3-6. Cumulative probability distribution of sIPSC amplitudes and interevent intervals in Tonic neurons treated with BSA or IL-1β.

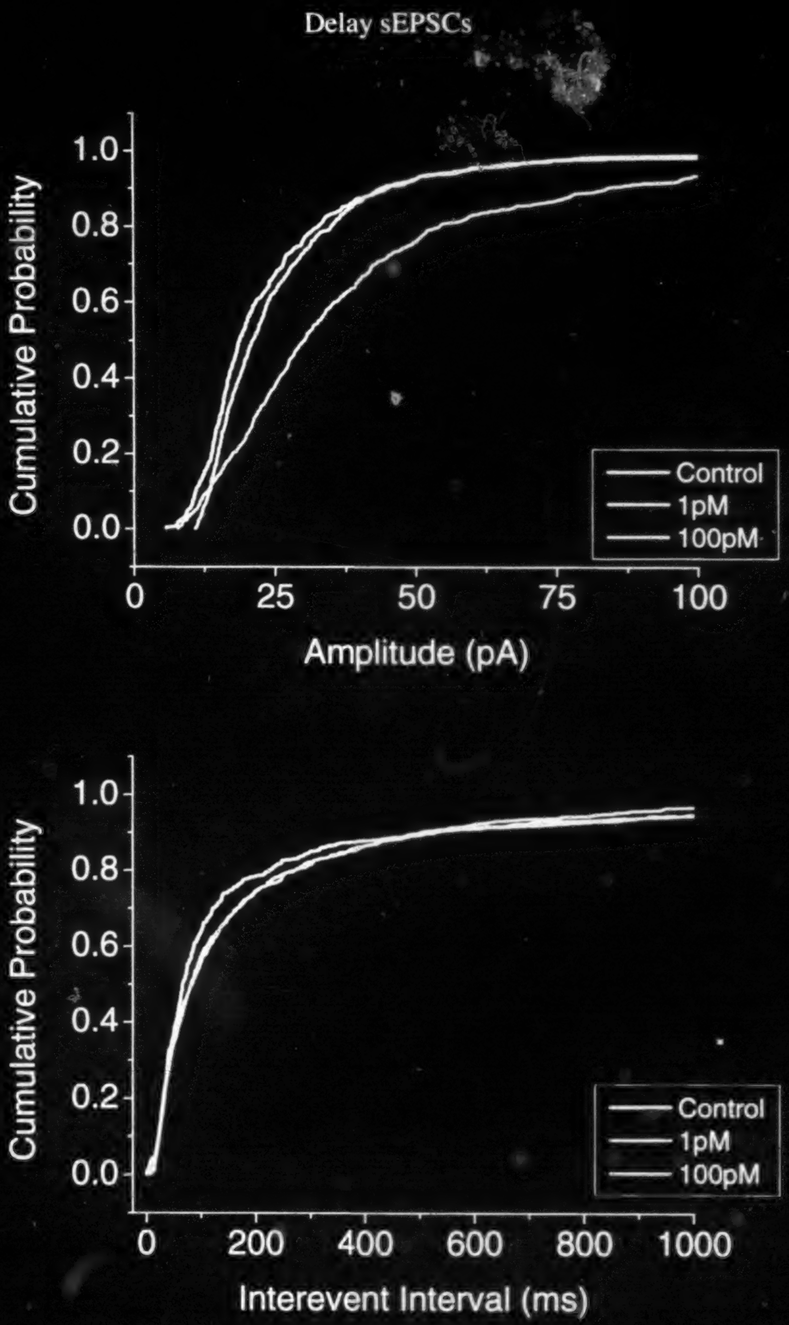


Figure 3-7. Cumulative probability distribution of sEPSC amplitudes and interevent intervals in Delay neurons treated with BSA or IL-1β.

Delay sIPSCs

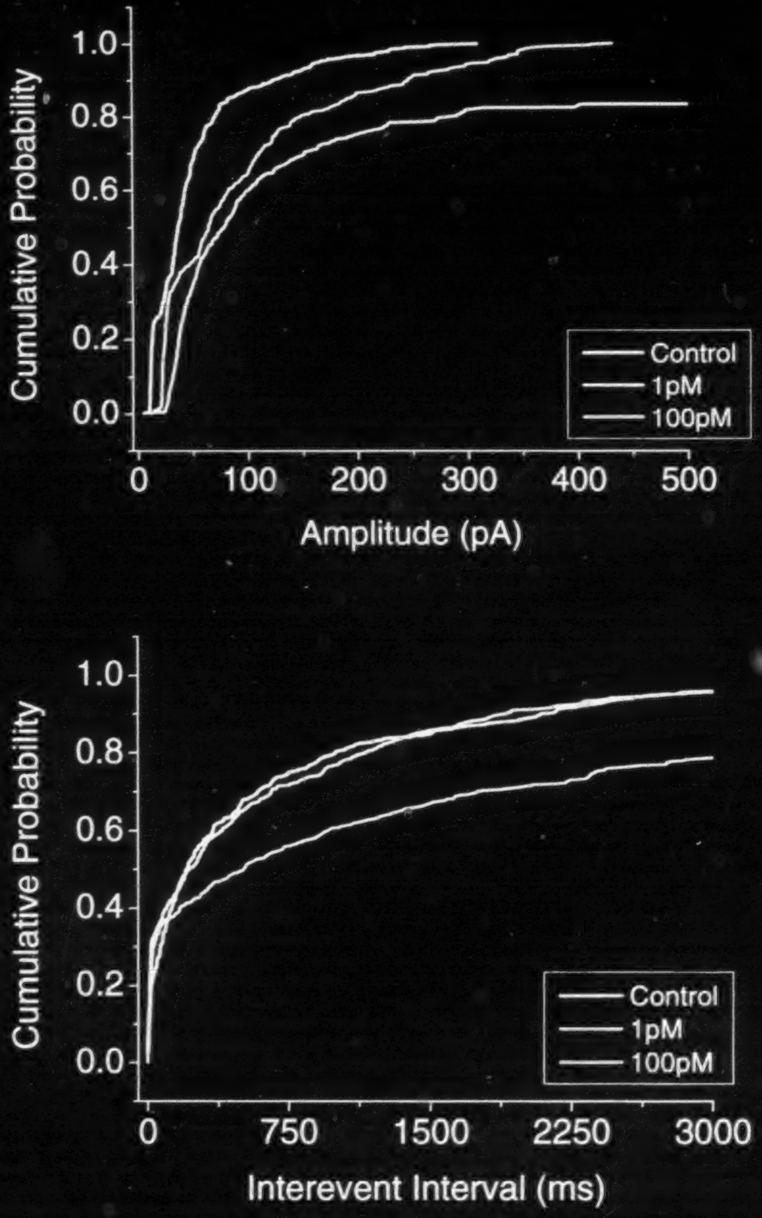
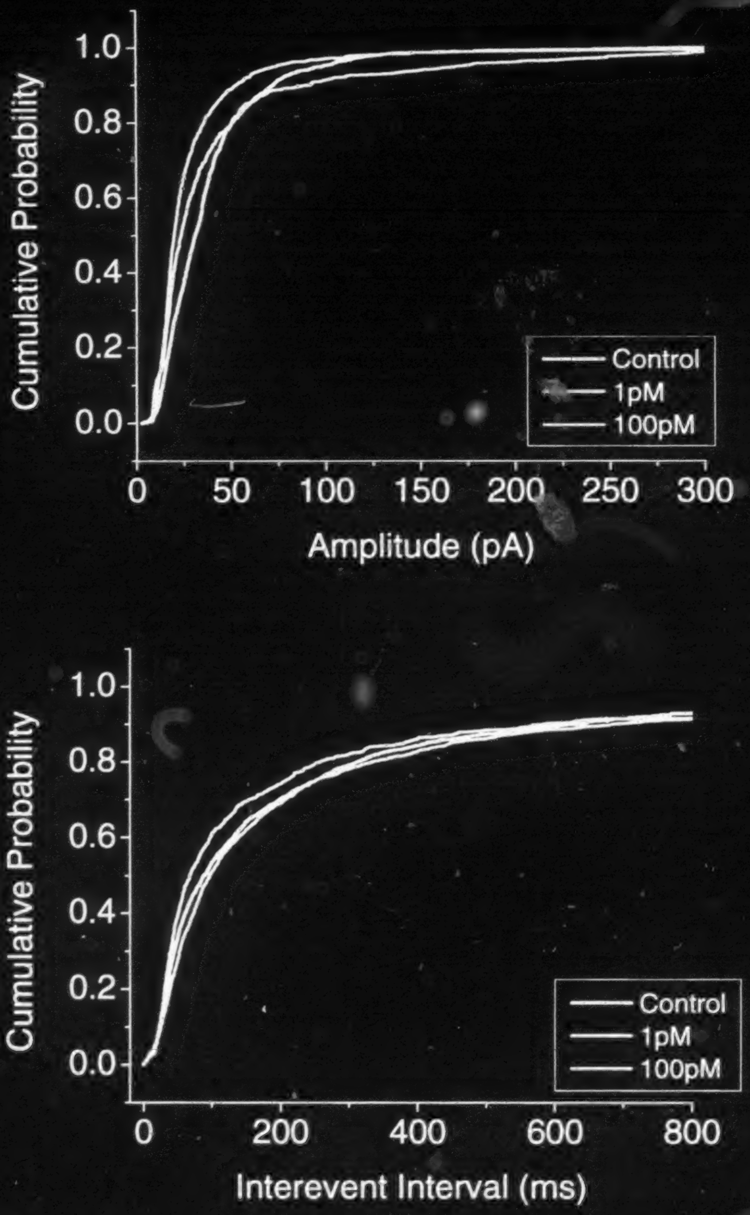
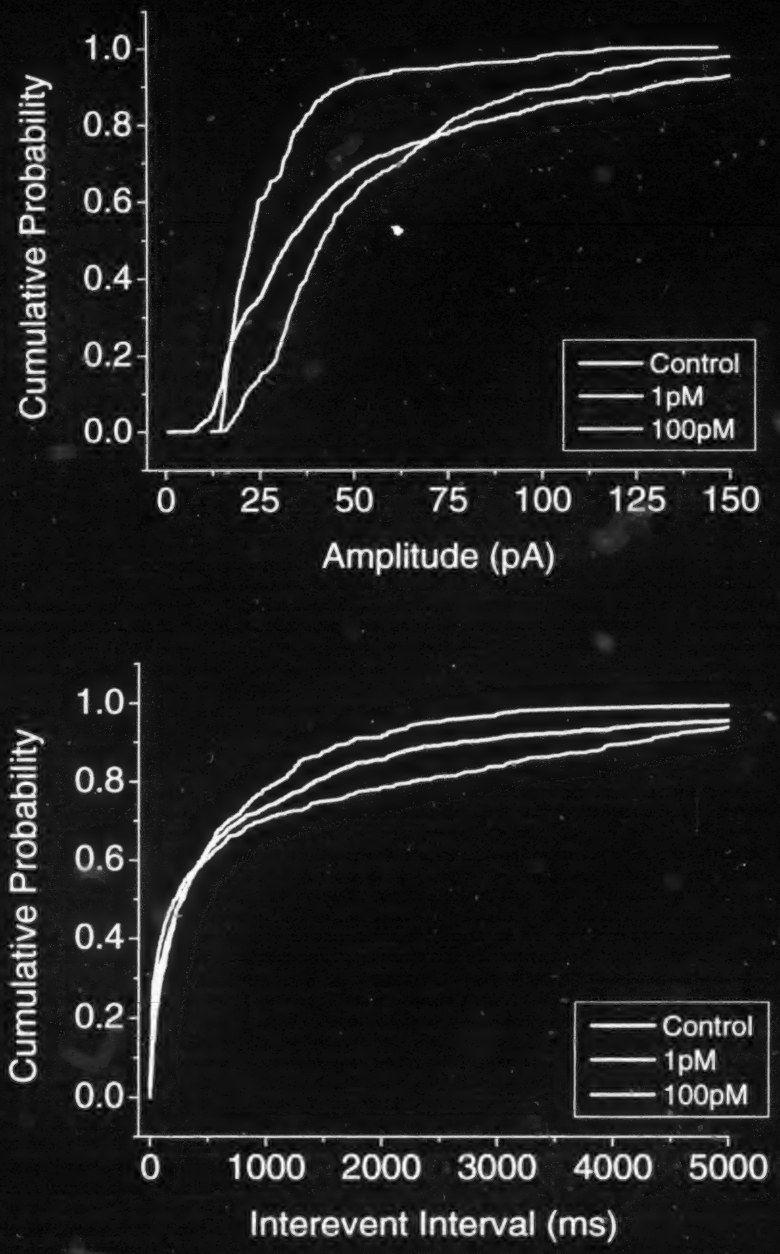


Figure 3-8. Cumulative probability distribution of sIPSC amplitudes and interevent intervals in Delay neurons treated with BSA or IL-1β.



Irregular sEPSCs

Figure 3-9. Cumulative probability distribution of sEPSC amplitudes and interevent intervals in Irregular neurons treated with BSA or IL-1β.



Irregular sIPSCs

Figure 3-10. Cumulative probability distribution of sIPSC amplitudes and interevent intervals in Irregular neurons treated with BSA or IL-1β.

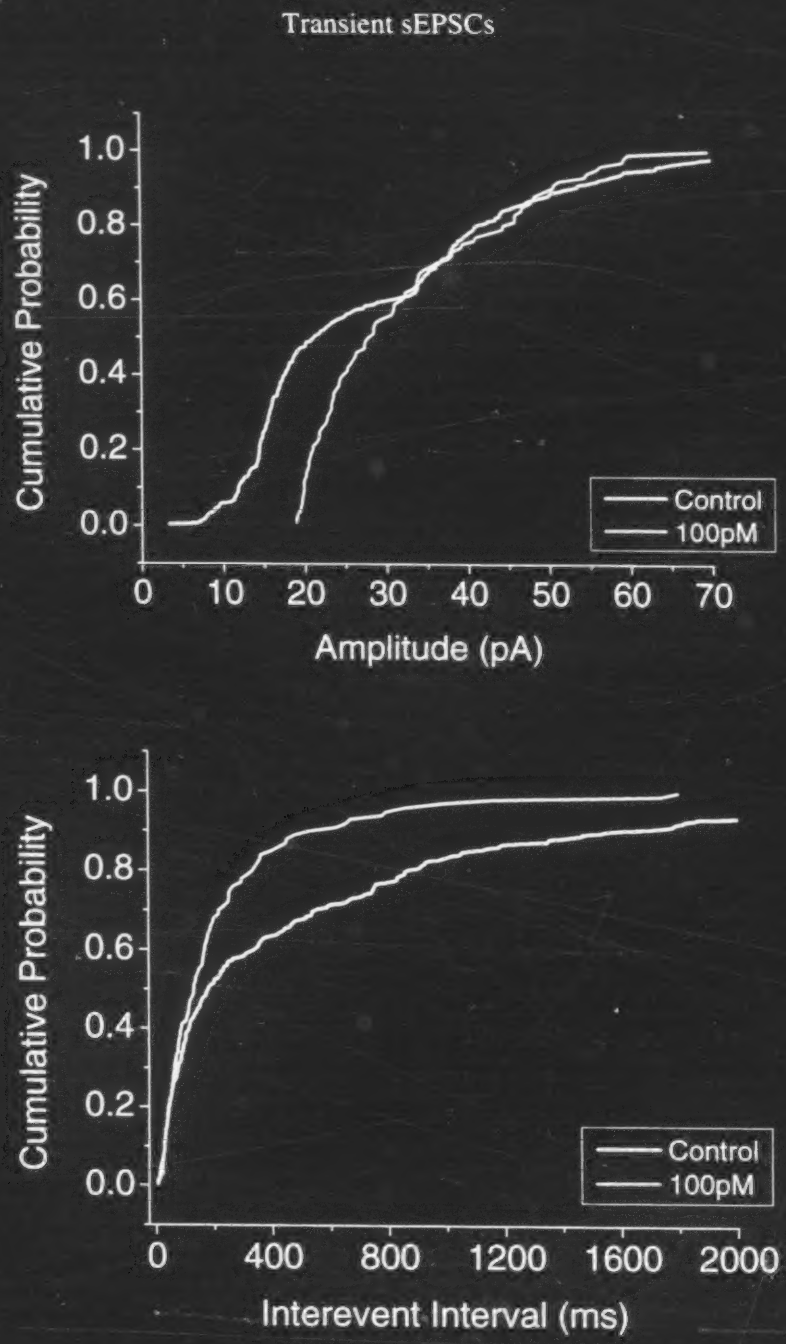

Transient sEPSCs

Figure 3-11. Cumulative probability distribution of sEPSC amplitudes and interevent intervals in Transient neurons treated with BSA or IL-1β.

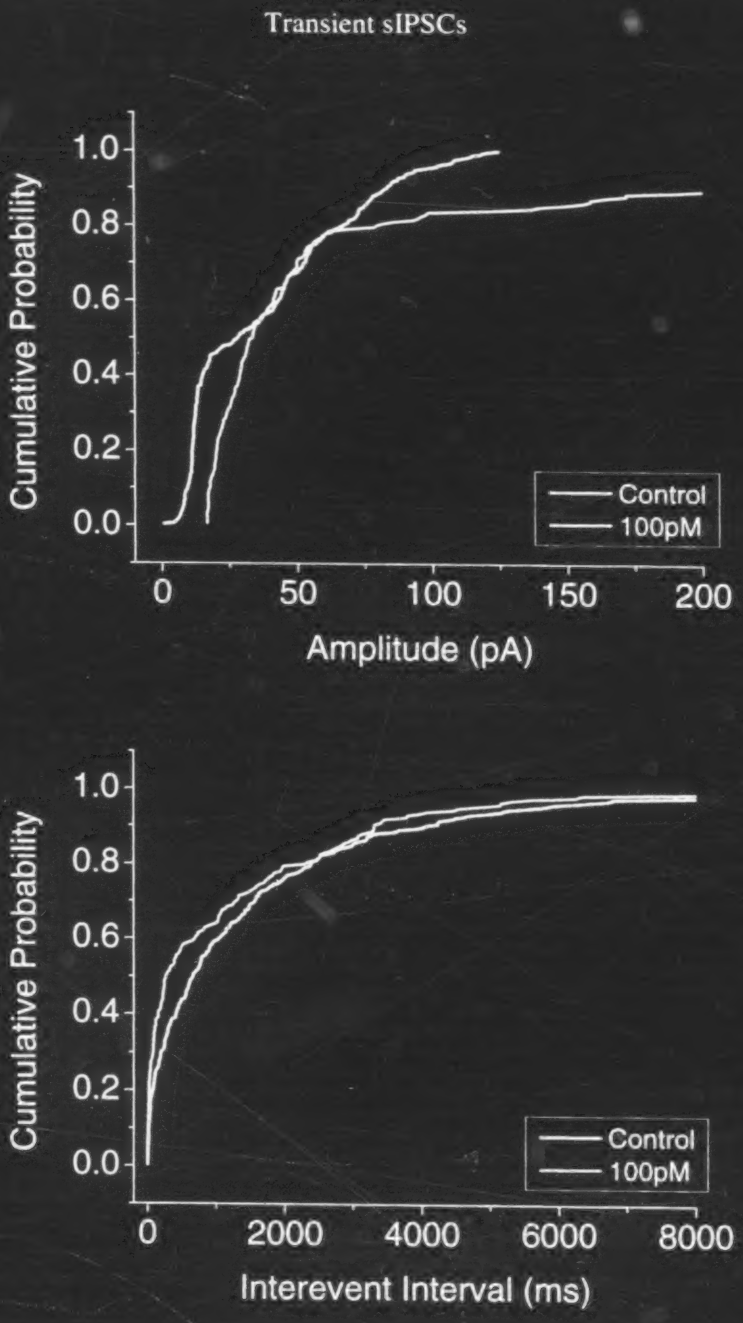


Figure 3-12. Cumulative probability distribution of sIPSC amplitudes and interevent intervals in Transient neurons treated with BSA or IL-1β.

Phasic sEPSCs

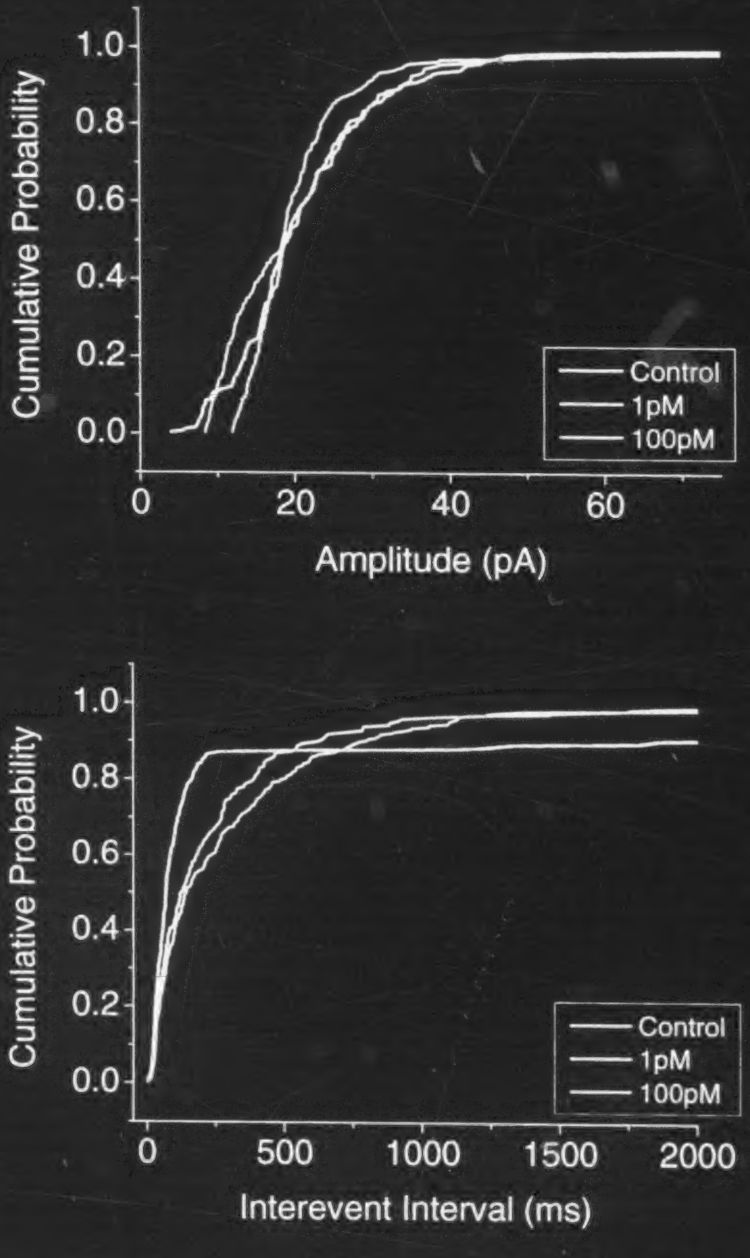


Figure 3-13. Cumulative probability distribution of sEPSC amplitudes and interevent intervals in Phasic neurons treated with BSA or IL-1β.

Phasic sIPSCs

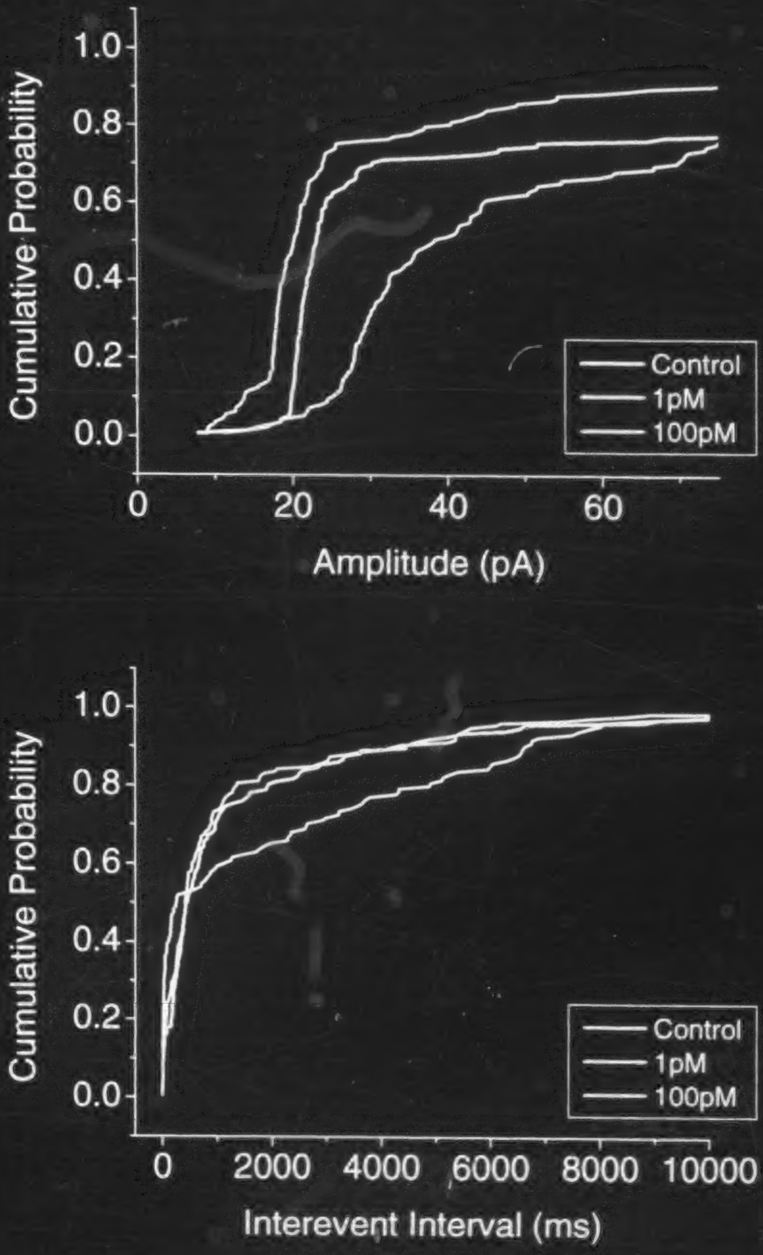


Figure 3-14. Cumulative probability distribution of sIPSC amplitudes and interevent intervals in Phasic neurons treated with BSA or IL-1β.

3.2 References

Lu, V.B., Moran, T.D., Balasubramanyan, S., Alier, K.A., Dryden, W.F., Colmers, W.F., & Smith, P.A. (2006). Substantia gelatinosa neurons in defined-medium organotypic slice culture are similar to those in acute slices from young adult rats. *Pain, 121*: 261-275.

CHAPTER 4 EFFECTS OF 1PM OF INTERLEUKIN-1BETA ON EXCITABILITY OF SPINAL DORSAL HORN NEURONS IN DMOTC

4.1 Effects of a Low Concentration (1pM) of IL-1β

The relative proportions of each cell type to the entire population of neurons was also described for the 1pM IL-β-treated condition (Figure 3-1). It was found that 28.0% were Tonic, 28.0% were Irregular, 36.0% were Delay, 4.0% were Transient, and 4.0% were Phasic. These proportions are relatively similar to those described for BSA controls and the high (100pM) concentration treatment condition, and to the proportions which have been previously reported in DMOTC cultures (Lu et al., 2006).

4.1.1 Resting Membrane Potential, Rheobase, Cell Capacitance

In general, the RMP, rheobase, and C_m of the five neuronal classes in the low concentration (1pM) of IL-1β condition were not significantly different than the corresponding neuronal types in the control group (p values ranged from 0.025 to 0.83; Figures 4-1, 4-2, and 4-3). Average RMPs ranged from −49mV to −70mV, average rheobase ranged from 33pA to 65pA, and average C_m ranged from 35pF to 48pF. Also, as was seen in the BSA controls, Tonic cells had the lowest average rheobase of all the neuronal types (31.667 pA; not statistically significant). All the other differences are summarized in Table 3-1.

The only statistically significant difference on any of the above measures between the neurons in the control condition and the cells treated with 1pM of IL-1β was the rheobase of Irregular cells (p=0.025).

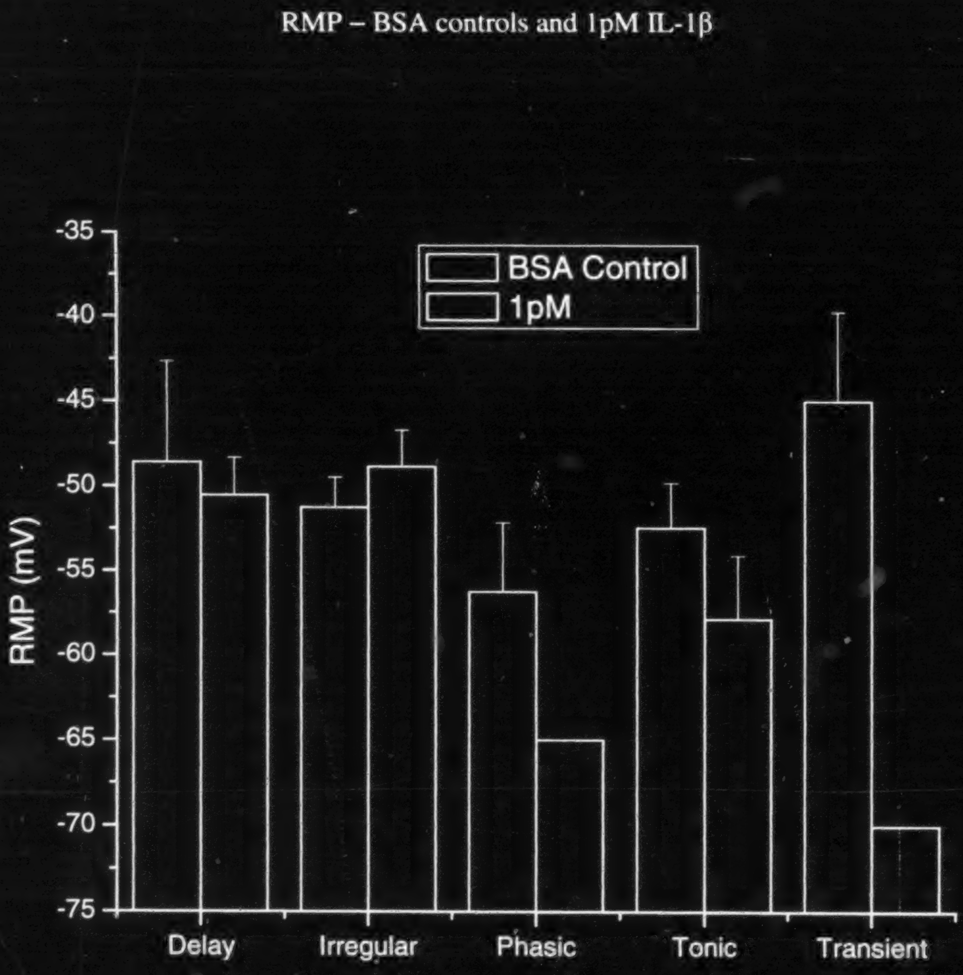
4.1.2 Spontaneous Post-Synaptic Potentials

The amplitudes and frequencies of excitatory and inhibitory spontaneous post-synaptic currents (sEPSCs and sIPSCs, respectively) were measured in both the BSA-treated control cells and the IL-1β-treated cells. The differences between sPSCs of IL-1β-treated cells and controls are summarized in Table 3-2.

With respect to sEPSCs, it was found that amplitudes were significantly greater following IL-1β treatment in Tonic cells and Delay cells (Figures 3-5 and 3-7). In contrast, amplitudes were significantly reduced following IL-1β treatment in Irregular cells, while sEPSC amplitudes were not significantly different in Phasic cells (p=0.07; Figures 3-9 and 3-13). sEPSC frequencies were significantly reduced following IL-1β treatment in Delay cells and Phasic cells, but significantly greater following IL-1β treatment in Irregular cells; lastly, in Tonic cells, there was no significant difference in sEPSC frequency (p=0.10; Figures 3-5, 3-7, 3-9, and 3-13).

In terms of sIPSCs, it was found that amplitudes were significantly greater following IL-1β treatment in Tonic cells, Delay cells, and Phasic cells, while in Irregular cells, amplitudes were significantly reduced (Figures 3-6, 3-8, 3-10, and 3-14). sIPSC frequencies were significantly reduced following IL-1β treatment in Tonic cells and Phasic cells, but significantly greater in Irregular cells and Delay cells (Figures 3-6, 3-8, 3-10, and 3-14).

Both increases and decreases in sEPSC and sIPSC amplitude were seen, as well as increases and decreases in sPSC frequency. However, these changes were different from those seen following treatment with 100pM IL-1β.



RMP – BSA controls and 1pM IL-1β

Figure 4-1. Comparison of resting membrane potential of BSA control neurons and neurons treated with 1pM IL-1β.

Rheobase – BSA controls and 1pM IL-1β

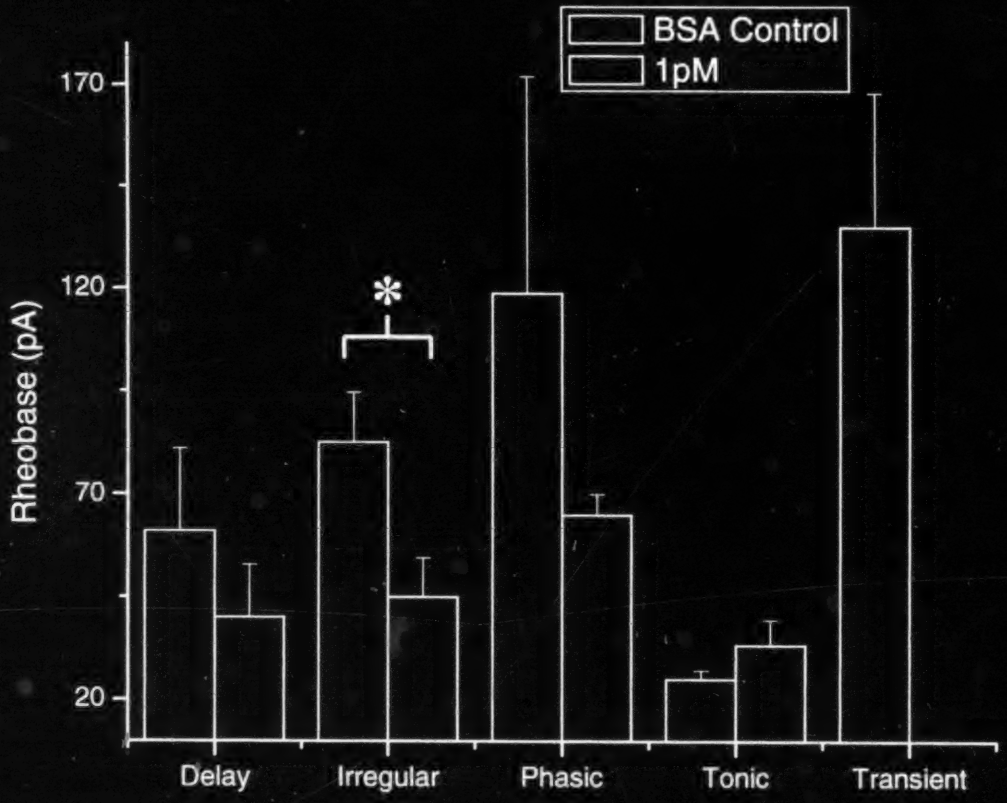


Figure 4-2. Comparison of rheobase of BSA control neurons and neurons treated with 1pM IL-1β.

C_m – BSA controls and 1pM IL-1β

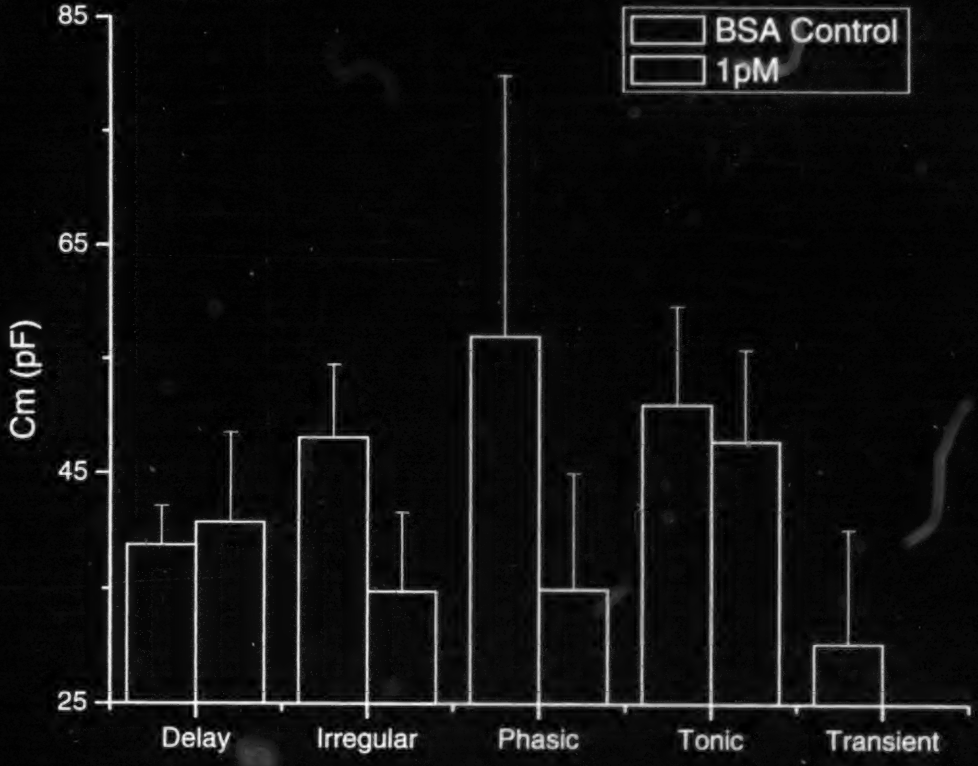


Figure 4-3. Comparison of cell capacitance of BSA control neurons and neurons treated with 1pM IL-1β.

4.2 References

Lu, V.B., Moran, T.D., Balasubramanyan, S., Alier, K.A., Dryden, W.F., Colmers, W.F., & Smith, P.A. (2006). Substantia gelatinosa neurons in defined-medium organotypic slice culture are similar to those in acute slices from young adult rats. *Pain, 121*: 261-275.

CHAPTER 5 ADDITIONAL ANALYSES AND DISCUSSION

5.1 Summary of Relevant Findings

When examining the sPSC results obtained with Kolmogorov-Smirnov statistics (cumulative probability plots), it became apparent that although a majority of the effects were significant, there was no clear direction of results; in fact, almost every possible change was seen. For example, amplitude changes in sPSCs were sometimes opposite in direction to frequency changes, sEPSC changes were not necessarily different from sIPSC changes, and effects on putative excitatory neurons (Delay) were neither the same nor the opposite to effects on putative inhibitory neurons (Tonic). Individually, these observations are not cause for concern nor do they suggest that the K-S test statistics are unfounded; however, collectively, these results did not yield any information regarding global effects of IL-1β or even mechanisms through which IL-1β may act. Thus, further experiments using calcium imaging were undertaken and the sPSC data were analyzed more conservatively in an effort to elucidate the most important effects of IL-1β.

5.2 Calcium Imaging

The presence of the calcium-sensitive dye Fluo-4 is an indicator of cellular Ca^{2+} concentrations, and by measuring cells' fluorescence in response to excitation via a high concentration of external K^+, it is possible to see whether slices treated with IL-1β are more or less excitable than control slices. Twenty-six cells from slices in each condition were analyzed, and both the amplitude and area under the curve of the K^+-induced fluorescence increase were measured for three different concentrations of K^+ (Figures 5-1, 5-2, and 5-3).

With respect to amplitude of the K^+-induced response, it was found that 100pM IL-1β-treated cells' response to 50mM K^+ was significantly greater than the amplitude of control cells' response (p=0.02). Similarly, the area under the curve of the 100pM IL-1β-treated cells' responses to 35mM and 50mM K^+ was significantly greater than the area of control cells' responses to both these concentrations (p=0.0076 and p=0.0009, respectively). These results suggest that treatment with 100pM IL-1β increases overall excitability.

In cells treated with 1pM IL-1β, it was found that these cells' response to 20mM K^+ was significantly lesser than the amplitude of control cells' response (p=0.008); these responses were not significantly different from control responses after 35mM or 50mM K^+. The area under the curve of the 1pm IL-1β-treated cells' responses was not significantly different from the area of control responses following any K^+ concentration. These results suggest that treatment with 1pM IL-1β has little effect on overall excitability.

5.3 sPSC Analysis - Means and Amplitude Histograms

The sPSC means were analyzed for amplitude and interevent interval of each cell type and each concentration of applied IL-1β. The results are summarized in Figures 5-4, 5-5, 5-6 and 5-7. Statistically comparing the mean changes with an unpaired t-test is generally considered to be more conservative than comparing the cumulative probability distributions with the K-S test; in fact, some would argue that the t-test misses important trends in the data. However, this analysis was done to gain a different perspective on the data and attempt to report effects that may be physiologically significant rather than those that are statistically significant. It must be stated that at this point, the data interpretation became more subjective in that only the effects that were found to be significant with both the K-S test and the unpaired t-test were examined. The assumption was made that events found to be statistically significant with only the K-S test may not necessarily be physiologically relevant.

5.3.1 sPSC Means

When examining sPSC means of the five neuronal types, it was found that many of the differences between controls and 100pM IL-1β-treated cells were not statistically significant according to a t-test, in spite of significance with the K-S test. The two cell types in which there were significant differences were sIPSC frequency in Delay cells and Irregular cells. Delay cells treated with 100pM IL-1β were found to have significantly lower sIPSC frequency than controls ($p=0.04$); in other words, inhibitory events in these cells were less frequent after treatment with a high concentration of IL-1β. Irregular cells treated with 100pM IL-1β were found to have significantly greater sIPSC frequency than controls ($p<0.01$); in other words, inhibitory events in these cells were more frequent after treatment with a high concentration of IL-1β.

In 1pM IL-1β-treated cells, there were only two cell types in which there were significant differences when compared to BSA controls; these were sIPSC amplitude in Irregular cells and sIPSC frequency in Tonic cells. Irregular cells treated with 1pM IL-1β were found to have significantly reduced sIPSC amplitude than controls ($p=0.05$); in other words, inhibitory events in these cells had a smaller amplitude after treatment with a low concentration of IL-1β. Tonic neurons treated with 1pM IL-1β were found to have significantly reduced sIPSC frequency than controls ($p=0.05$); in other words, inhibitory events in these cells were less frequent after treatment with a low concentration of IL-1β.

From this analysis, four effects were found to be significant. To narrow down which effects were significant at which concentration, the K-S test data was re-examined, and only the effects that appeared to be extremely significant on the cumulative probability plots ($p<0.0036$) were retained; these eleven events included the four from the sPSC means. From these eleven effects, only the effects on Tonic cells and on Delay cells were considered, since these have been characterized much more extensively than any other cell type: Tonic cells are thought to be inhibitory, while Delay cells are thought to be excitatory (Balasubramanyan, Stemkowski, Stebbing, & Smith, 2006). Thus, there were eight large, quite significant effects observed following application of IL-1β, and these were:

1) an increase in sEPSC amplitude in Tonic cells after treatment with 1pM IL-1β
2) an increase in sIPSC amplitude in Tonic cells after treatment with 1pM IL-1β
3) a decrease in sIPSC frequency in Tonic cells after treatment with 1pM IL-1β
4) an increase in sIPSC amplitude in Delay cells after treatment with 1pM IL-1β
5) an increase in sEPSC amplitude in Delay cells after treatment with 100pM IL-1β
6) a decrease in sEPSC frequency in Delay cells after treatment with 100pM IL-1β
7) a decrease in sIPSC frequency in Tonic cells after treatment with 100pM IL-1β
8) a decrease in sIPSC frequency in Delay cells after treatment with 100pM IL-1β

These results are summarized in Table 5-1 and in Figures 5-8 and 5-9.

5.3.2 sPSC Amplitude Histograms

In an effort to distinguish between populations of individual spontaneous events, amplitude histograms were plotted for each of the big effects. Interevent interval data were not considered, since time interval between events is best fit with a monoexponential equation (Gordon & Bains, 2005), thus it would be impossible to visualize different populations of events. Of the four amplitude changes considered, three were following treatment with the low concentration (1pM) of IL-1β and one was following treatment with the high concentration (100pM) of IL-1β. All sPSC amplitudes increased following treatment with IL-1β, which might reflect an increase in pre-synaptic quantal content or a change in pre- or post-synaptic neurotransmitter receptors.

After treatment with 100pM IL-1β, sEPSC amplitudes of Delay neurons increased (Figure 5-10). More specifically, there were fewer low-amplitude events (between 1 and 50 pA) and many more high-amplitude events (larger than 100pA). Since Delay cells are putative excitatory neurons, this suggests the cells were more excitable following treatment with 100pM IL-1β.

The major amplitude changes after treatment with 1pM IL-1β were as follows: Tonic cells' sEPSC and sIPSC amplitudes increased (Figures 5-11 and 5-12), and Delay cells' sIPSC amplitudes increased (Figure 5-13). With respect to Tonic sEPSCs, there were fewer low-amplitude events (between 1 and 50 pA) and more high-amplitude events (larger than 100pA), as was seen in Delay cells after 100pM IL-1β. The same trends were seen in sIPSCs of Tonic and Delay cells: after treatment with 1pM IL-1β, there was a rightward shift in the amplitude histograms. In the sIPSCs, it should be noted that events larger than 300pA were extremely rare in the control condition, but after treatment with 1pM IL-1β, more extremely high-amplitude (larger than 300pA) events were seen. Increases in Tonic sEPSC and Delay sIPSC amplitudes suggest that cells were less excitable following treatment with 1pM IL-1β.

Table 5-1

Summary of Very Apparent Significant Differences in Spontaneous Post-Synaptic Currents (sPSCs) of IL-1β-Treated Cells When Compared With BSA Controls

	TONIC		**DELAY**	
1pM IL-β sEPSCs	**Amplitude** ↑	*Frequency* *NS*	*Amplitude* ↑	*Frequency* ↓
1pM IL-β sIPSCs	**Amplitude** ↑	**Frequency** ↓	**Amplitude** ↑	*Frequency* ↓
100pM IL-β sEPSCs	*Amplitude* ↑	*Frequency* *NS*	**Amplitude** ↑	**Frequency** ↓
100pM IL-β sIPSCs	*Amplitude* ↓	**Frequency** ↓	*Amplitude* ↑	**Frequency** ↓

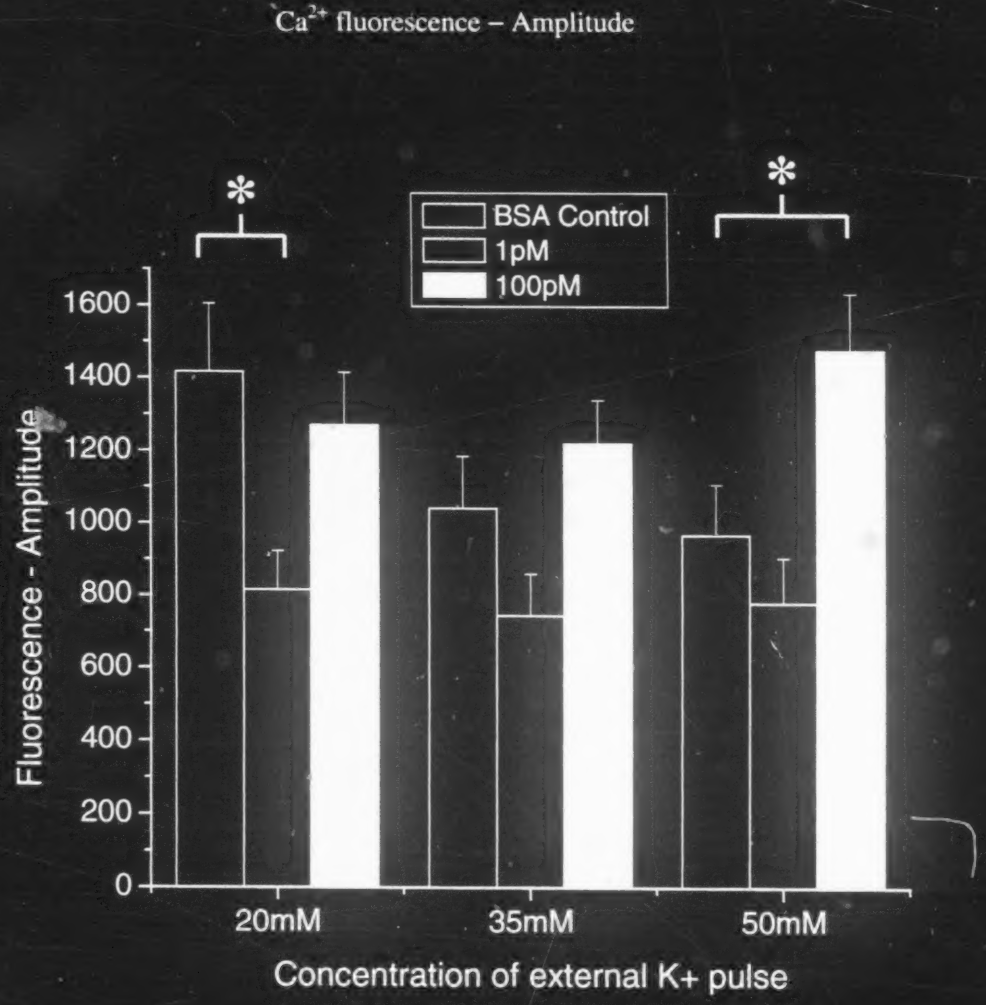
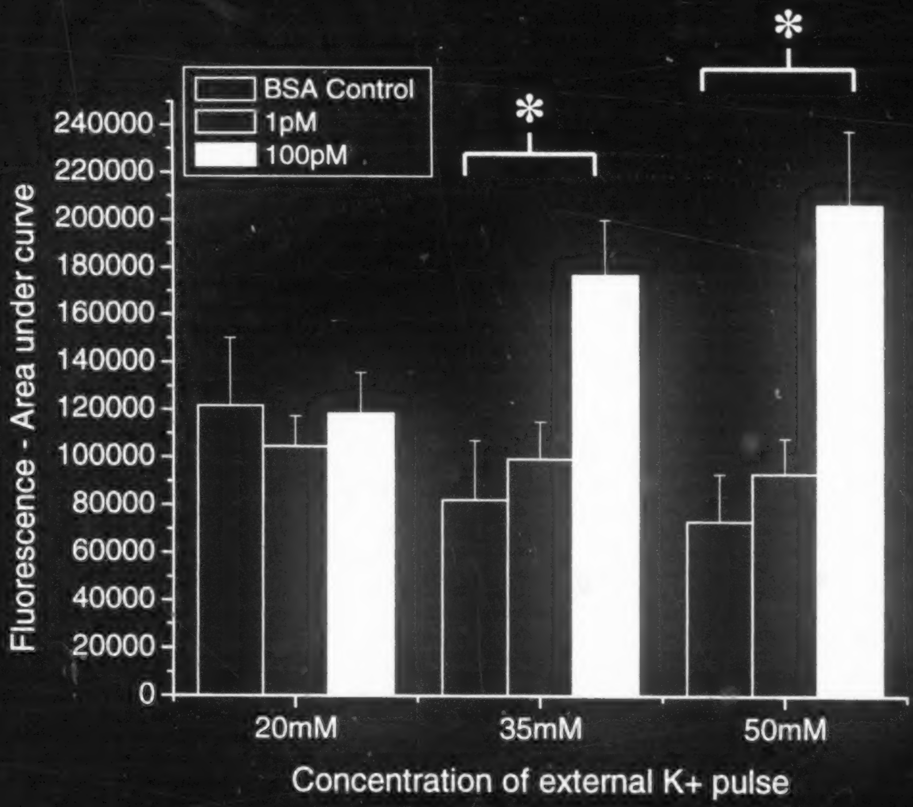


Figure 5-1. Summary of differences by treatment condition in Ca^{2+} fluorescence amplitudes in response to various concentrations of external K^{+}.



Ca^{2+} fluorescence – Area under the response curve

Figure 5-2. Summary of differences by treatment condition in Ca^{2+} fluorescence area under the response curve following various concentrations of external K^{+}.

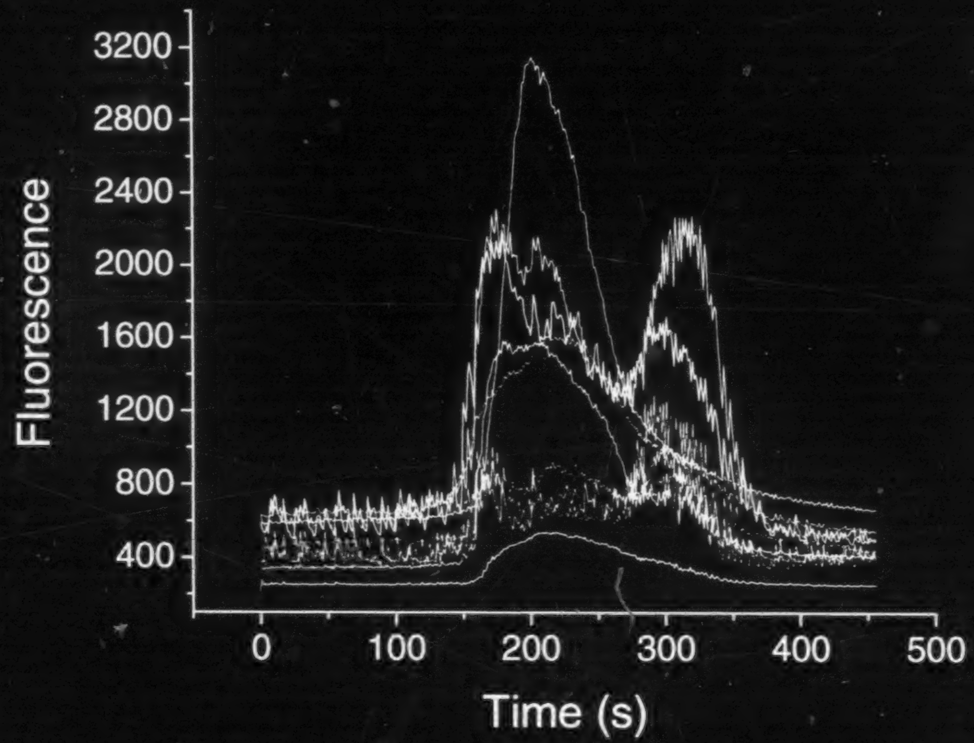


Figure 5-3. Fluorescence of dorsal horn neurons (various regions of interest in different colours) in response to application of 20mM KCl via external bath solution.

sEPSC means – BSA controls and 100pM IL-1β

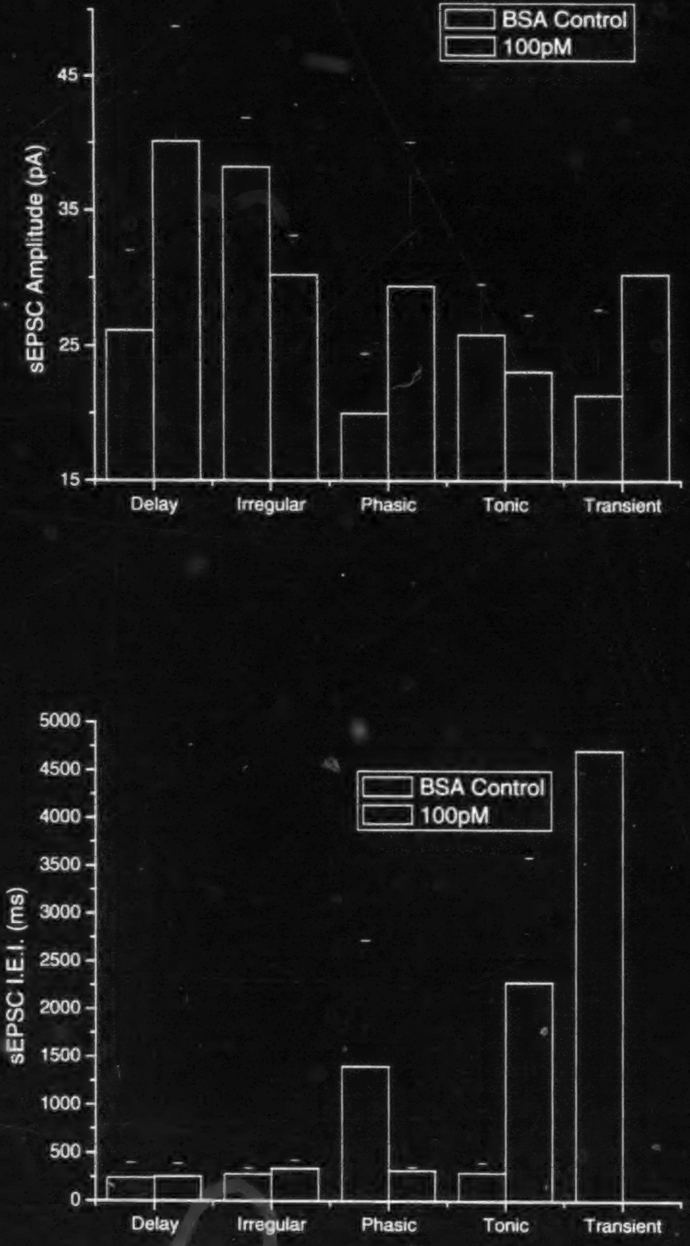


Figure 5-4. Means of sEPSC amplitudes and interevent intervals for BSA control cells and cells treated with 100pM IL-1β.

sIPSC means – BSA controls and 100pM IL-1β

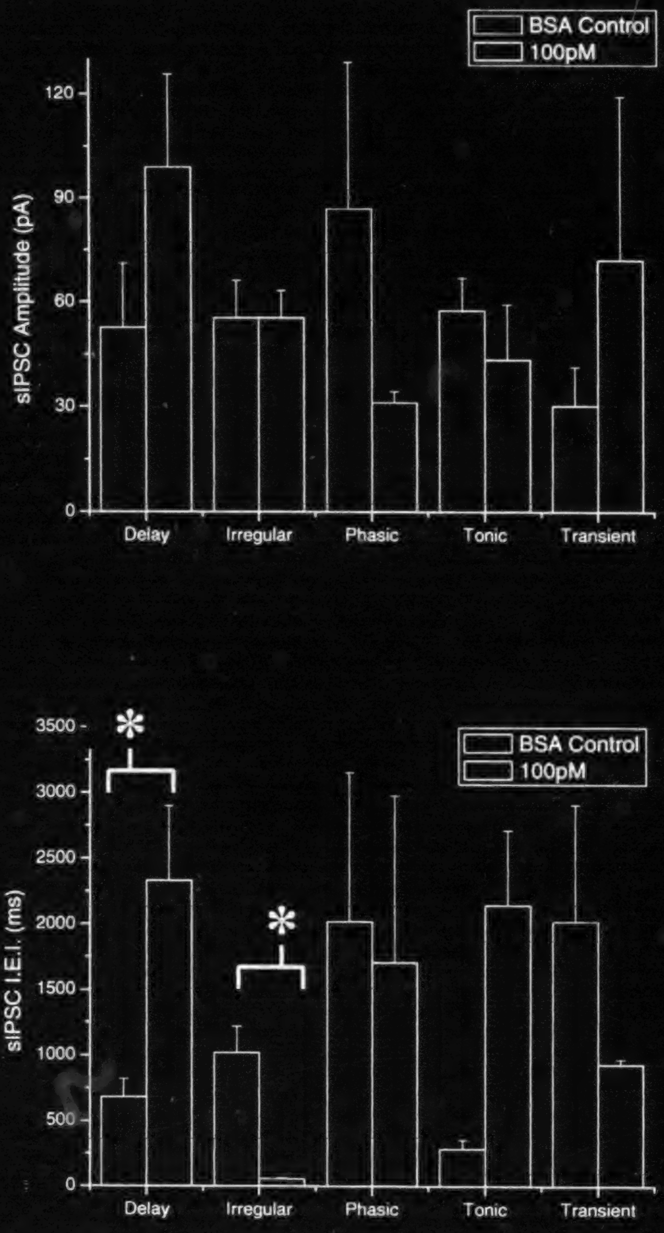
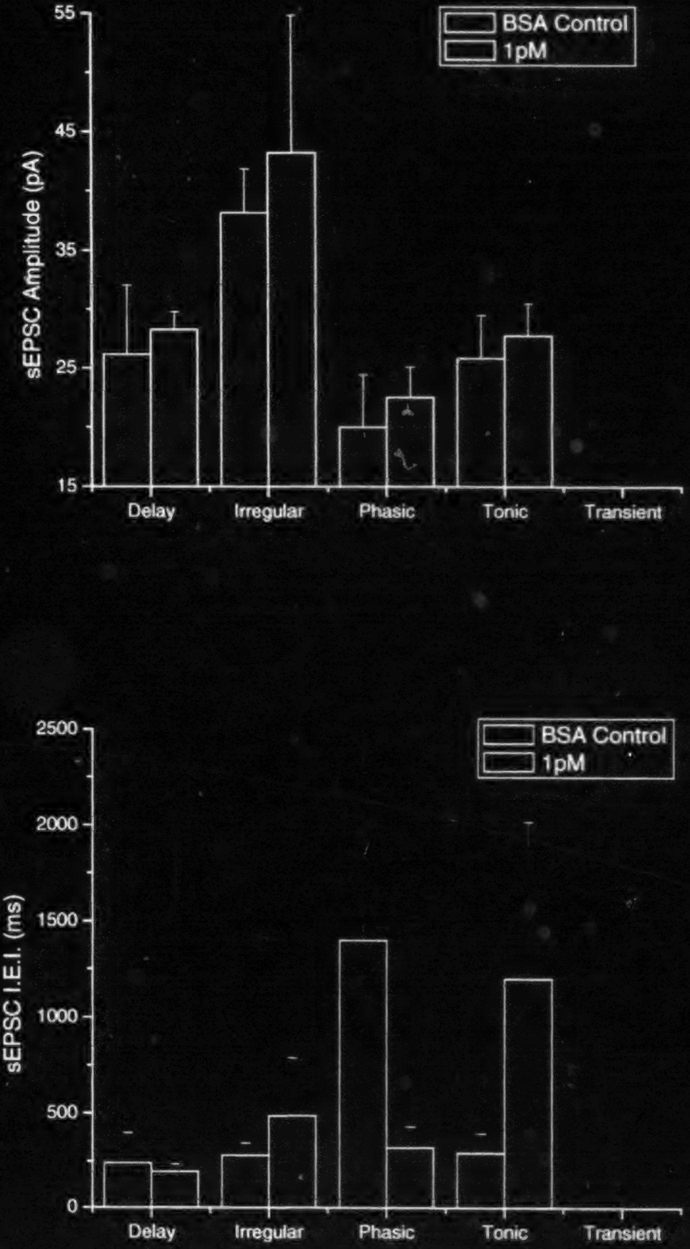


Figure 5-5. Means of sIPSC amplitudes and interevent intervals for BSA control cells and cells treated with 100pM IL-1β.



sEPSC means – BSA controls and 1pM IL-1β

Figure 5-6. Means of sEPSC amplitudes and interevent intervals for BSA control cells and cells treated with 1pM IL-1β.

sIPSC means – BSA controls and 1pM IL-1β

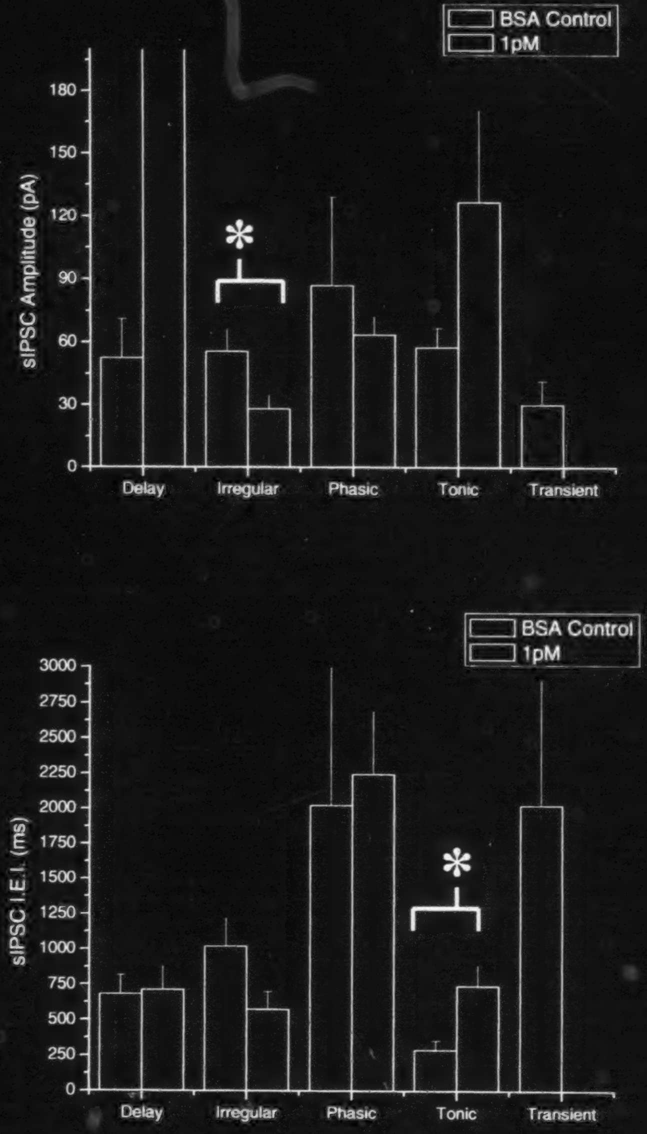


Figure 5-7. Means of sIPSC amplitudes and interevent intervals for BSA control cells and cells treated with 1pM IL-1β.

Very apparent significant differences after 100pM IL-1β

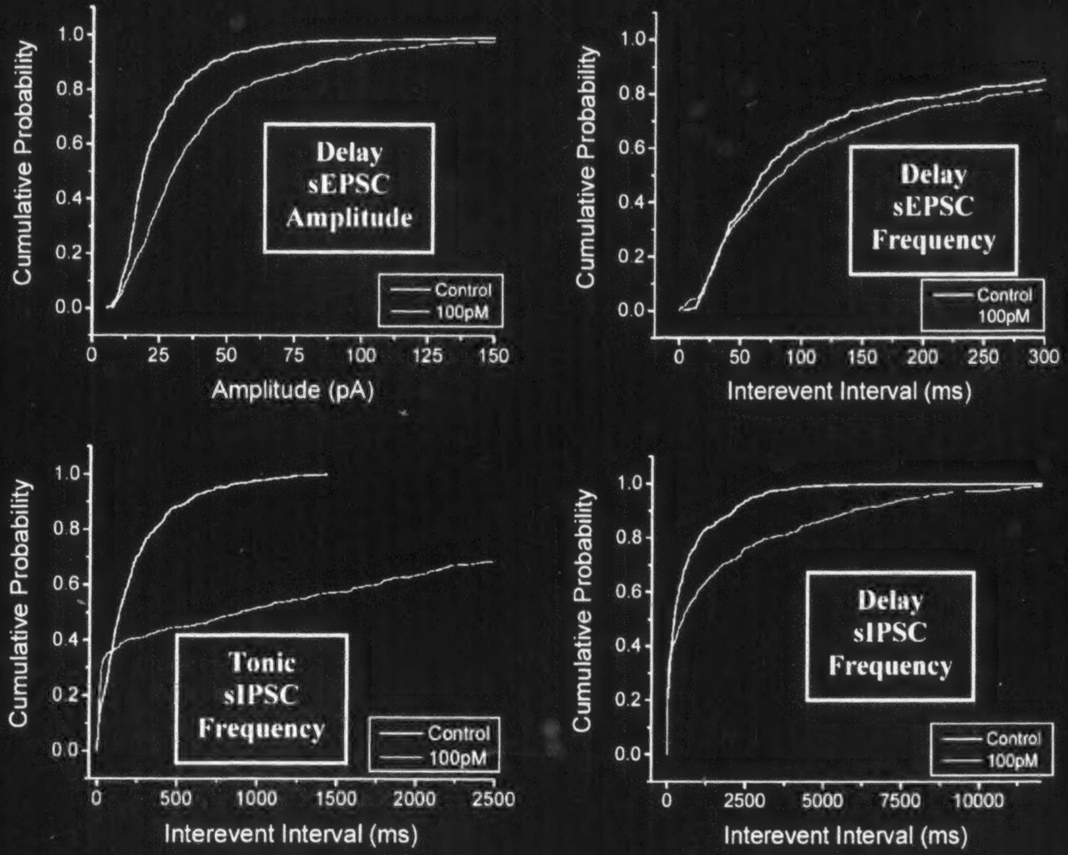


Figure 5-8. Cumulative probability distributions for the most apparent significant differences after treatment with 100pM IL-1β.

Very apparent significant differences after 1pM IL-1β

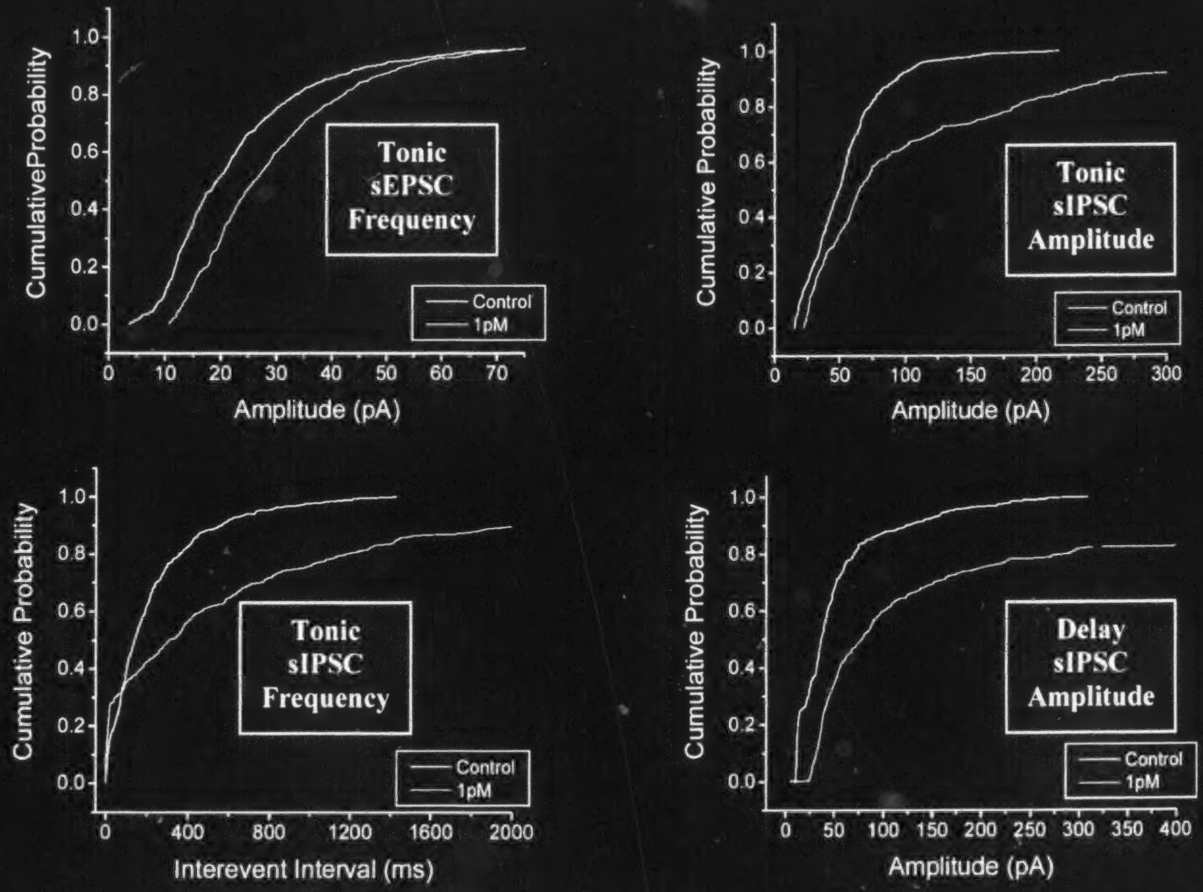
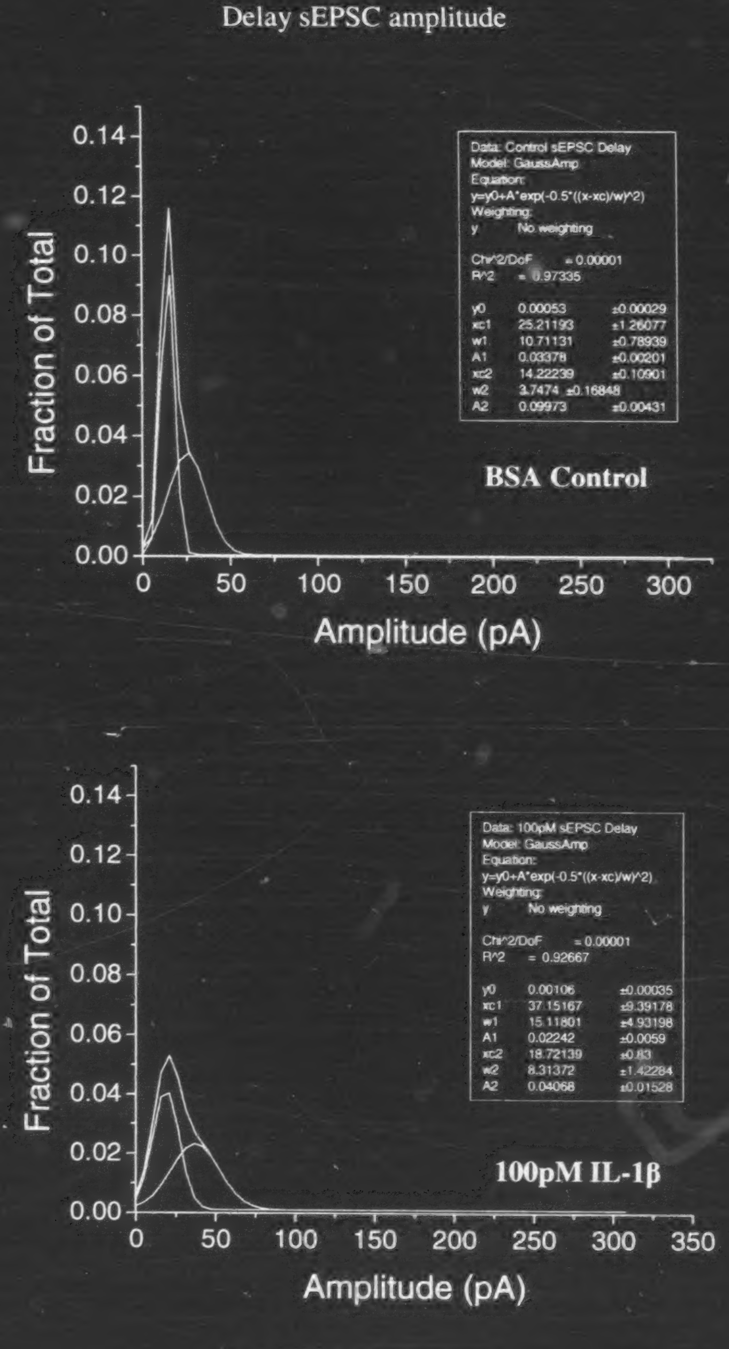


Figure 5-9. Cumulative probability distributions for the most apparent significant differences after treatment with 1pM IL-1β.



Delay sEPSC amplitude

```
Data: Control sEPSC Delay
Model: GaussAmp
Equation:
y=y0+A*exp(-0.5*((x-xc)/w)^2)
Weighting:
y         No weighting

Chi^2/DoF       = 0.00001
R^2      = 0.97335

y0       0.00053          ±0.00029
xc1      25.21193         ±1.26077
w1       10.71131         ±0.78939
A1       0.03378          ±0.00201
xc2      14.22239         ±0.10901
w2       3.7474  ±0.16848
A2       0.09973          ±0.00431
```

BSA Control

```
Data: 100pM sEPSC Delay
Model: GaussAmp
Equation:
y=y0+A*exp(-0.5*((x-xc)/w)^2)
Weighting:
y         No weighting

Chi^2/DoF       = 0.00001
R^2      = 0.92667

y0       0.00106          ±0.00035
xc1      37.15167         ±9.39178
w1       15.11801         ±4.93198
A1       0.02242          ±0.0059
xc2      18.72139         ±0.83
w2       8.31372          ±1.42284
A2       0.04068          ±0.01528
```

100pM IL-1β

Figure 5-10. Amplitude histogram for sEPSCs in Delay cells following treatment with 100pM IL-1β.

Tonic sEPSC amplitude

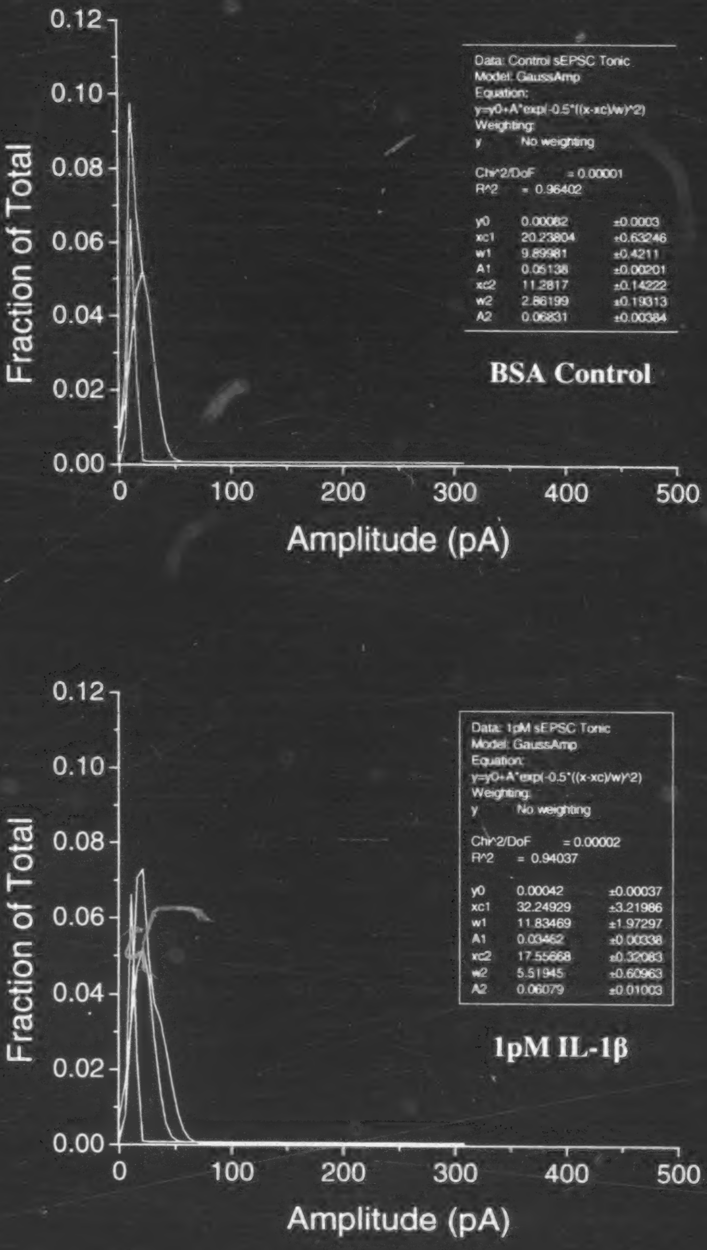
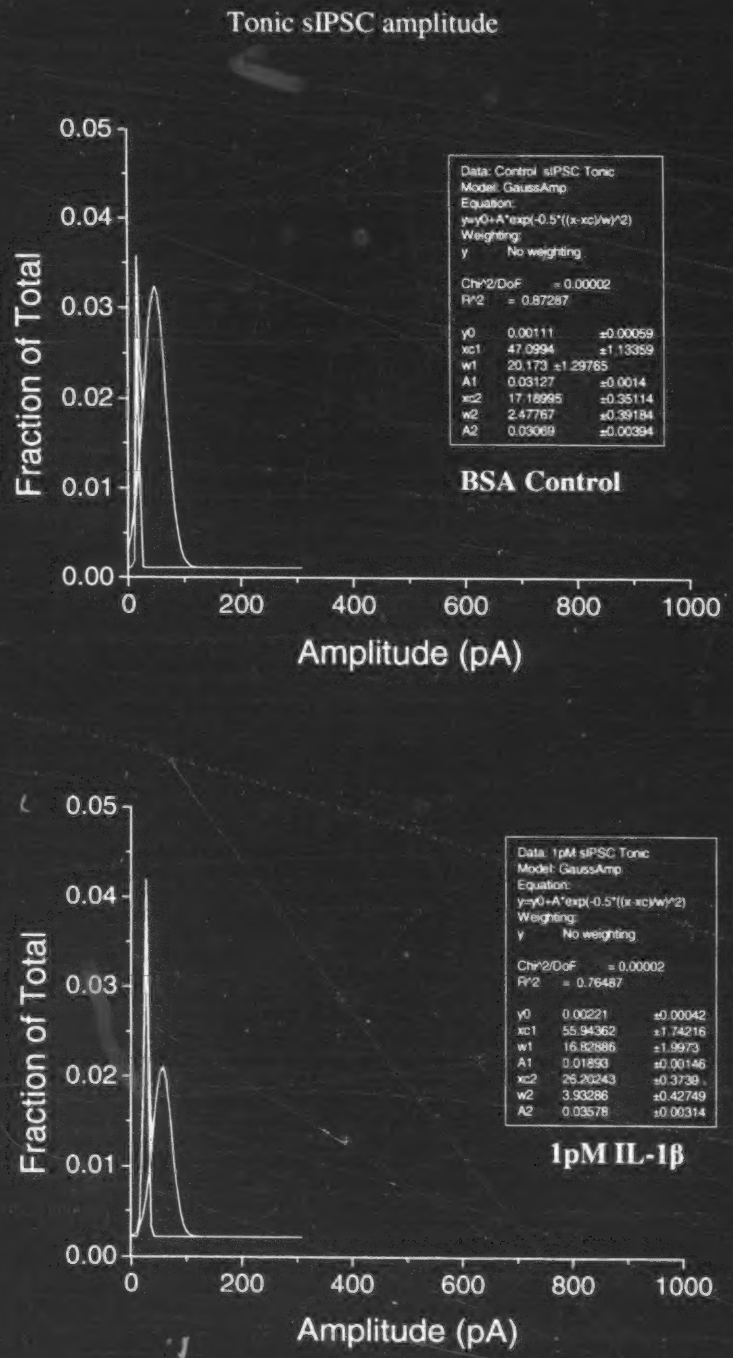


Figure 5-11. Amplitude histogram for sEPSCs in Tonic cells following treatment with 1pM IL-1β.



```
Data: Control sIPSC Tonic
Model: GaussAmp
Equation:
y=y0+A*exp(-0.5*((x-xc)/w)^2)
Weighting:
y         No weighting

Chi^2/DoF       = 0.00002
R^2     =  0.87287

y0      0.00111           ±0.00059
xc1     47.0994           ±1.13359
w1      20.173  ±1.29765
A1      0.03127           ±0.0014
xc2     17.18995          ±0.35114
w2      2.47767           ±0.39184
A2      0.03069           ±0.00394
```

```
Data: 1pM sIPSC Tonic
Model: GaussAmp
Equation:
y=y0+A*exp(-0.5*((x-xc)/w)^2)
Weighting:
y         No weighting

Chi^2/DoF       = 0.00002
R^2     =  0.76487

y0      0.00221           ±0.00042
xc1     55.94362          ±1.74216
w1      16.82886          ±1.9973
A1      0.01893           ±0.00146
xc2     26.20243          ±0.3739
w2      3.93286           ±0.42749
A2      0.03578           ±0.00314
```

Figure 5-12. Amplitude histogram for sIPSCs in Tonic cells following treatment with 1pM IL-1β.

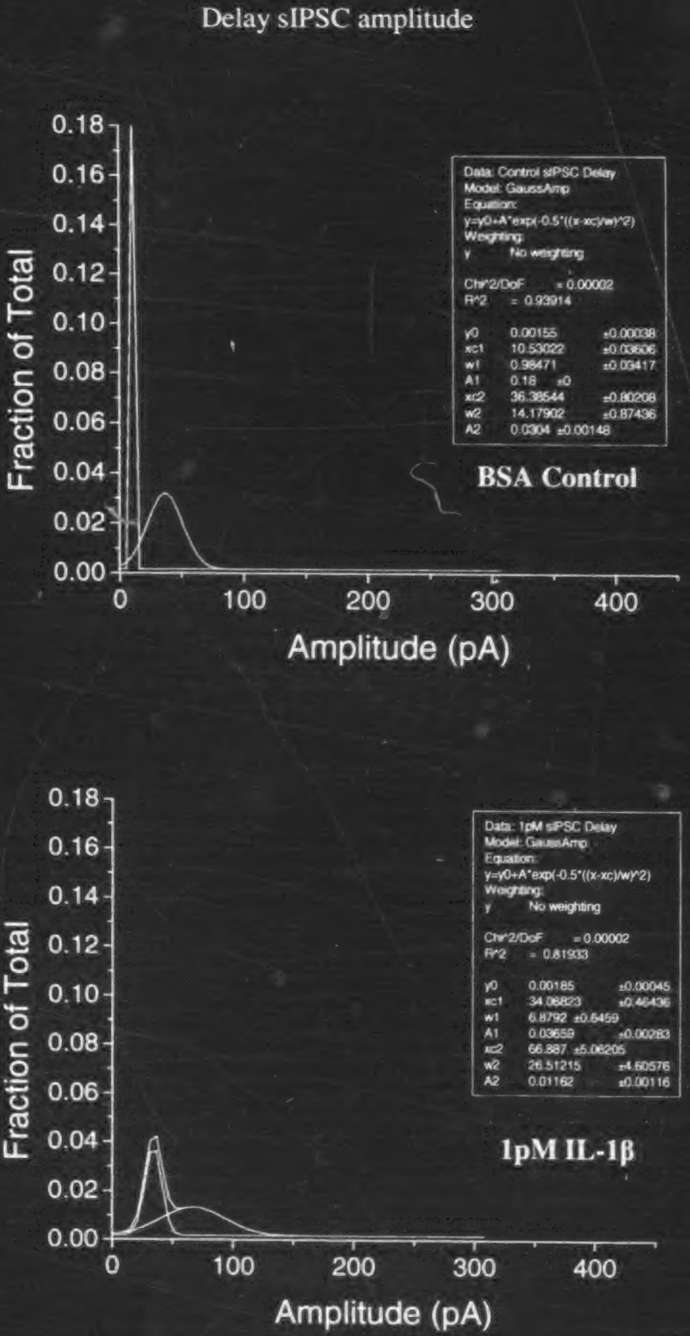


Figure 5-13. Amplitude histogram for sIPSCs in Delay cells following treatment with 1pM IL-1β.

5.4 *References*

Balasubramanyan, S., Stemkowski, P.L., Stebbing, M.J., & Smith, P.A. (2006). Sciatic chronic constriction injury produces cell-type-specific changes in the electrophysiological properties of rat substantia gelatinosa neurons. *J Neurophysiol, 96*: 579-590.

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CHAPTER 6 GENERAL DISCUSSION AND CONCLUSIONS

6.1 Summary of Results

The main finding of this thesis project was that long-term application of IL-1β produces significant, cell-type-specific changes in neurons of the spinal dorsal horn. Results from calcium-imaging experiments suggest that a low concentration (1pM) of IL-1β has little effect on overall excitability, while a high concentration (100pM) of IL-1β increases excitability in the dorsal horn. Results from the largest eight sPSC changes are similar, but not as definite: it appears as though 1pM IL-1β may act to decrease excitatory transmission and increase inhibitory transmission in the dorsal horn, while 100pM IL-1β may do the opposite: increase excitatory transmission while decreasing inhibitory transmission. This conclusion is based on the observation in acute slices that Tonic neurons tend to be inhibitory neurons, while Delay neurons are most likely excitatory.

Of the largest sPSC effects, there are some other important observations to be made. First of all, 1pM IL-1β had the largest impact on Tonic cells, increasing sEPSC and sIPSC amplitude while decreasing sIPSC frequency. Also, three of the four main effects were changes in amplitude. On the other hand, 100pM seemed to change mainly the properties of Delay cells, increasing sEPSC amplitude and decreasing sEPSC and sIPSC frequency. In this condition, three of the four main effects following 100pM IL-1β treatment were changes in frequency. This may indicate that different mechanisms are at work after treatment with the different concentrations of IL-1β, such that a lower concentration exerts its effects on inhibitory neurons and acts pre- and post-synaptically to alter neuronal properties, while a higher concentration affects excitatory neurons and likely acts pre-synaptically to increase excitability in the dorsal horn.

6.2 *Discussion of ELISA Results and Concentrations in Literature*

A considerable amount of literature is available concerning the actions of IL-1β, and a variety of concentration-dependent effects have been observed. A study conducted by Desson & Ferguson (2003) showed that in CNS circumventricular organs, IL-1β could produce a variety of dose-dependent effects on neuronal electrophysiological properties. They classified concentrations of 1pM, 500fM, and 100fM as being physiological (subseptic), while concentrations above 1nM were pharmacological (septic) concentrations. Based on these and other findings, the intention of the current project was to employ two concentrations of IL-1β, in hopes that the lower concentration (1pM) would represent an injured state and produce changes characteristic of overall neuronal hyperalgesia, while the higher concentration (100pM) would either result in the same effects on a larger scale, or invoke autoregulatory mechanisms and produce neuronal changes in line with overall analgesia, as there is strong evidence that IL-1β has a bell-shaped dose-response curve (Zelenka, Schäfers, & Sommer, 2005).

The measured concentrations as determined by ELISA of the two treatment conditions in the current project were found to be 32pM (following treatment with 1pM) and 118pM (following treatment with 100pM). This result is not completely unexpected, since the application of IL-1β should induce pro-inflammatory cytokine cascades that result in the amplification of the IL-1β signal (Inoue, 2006), and the ELISA measurements were taken nine days following treatment with IL-1β. The measured concentration of IL-1β after nine days was much greater in the 1pM treatment group (32-fold greater) than in the 100pM group (1.2-fold greater); the latter may reflect the involvement of negative-feedback control mechanisms which have been reported in other studies following treatment with high concentrations of IL-1β (Bernardino, Xapelli, Silva, Jakobsen, Poulsen, Oliveira et al., 2005; Zelenka et al., 2005). Lastly, it is always important to consider that the effective concentrations of IL-1β at the time of data collection cannot be quantified unequivocally: IL-1β is inevitably degraded by peptidases, there may be local release of IL-1β by microglia, and IL-1β may bind to nonreceptor binding sites (Bernardino et al., 2005).

Thirty-two picomolar and 118pM correspond to 554 pg/mL and 2041 pg/mL, respectively. These concentrations are higher than those which elicited NMDA receptor-mediated changes in intracellular Ca^{2+} concentration in hippocampal neurons in culture: Viviani et al. (2003) showed that 25-100 pg/mL IL-1β increased NMDA-induced intracellular Ca^{2+}, reaching a maximal effect at 50 pg/mL, while higher concentrations of IL-1β (250-500 pg/mL) were ineffective, and 1000 pg/mL IL-1β was slightly inhibitory. On the other hand, another study using hippocampal neurons conducted by Lai et al. (2006) reported no observable changes in surface expression of AMPA receptors at IL-1β concentrations lower than 1000 pg/mL, yet found significant decreases in the surface expression of GluR1 in the presence of 10 000-50 000 pg/mL. These concentrations are much higher than those in the current project, and when compared to other studies in the brain that employ IL-1β, it seems the findings reported by Lai et al. (2006) may reflect pharmacological effects that would never be seen in vivo.

A potential concern in the study of IL-1β is the diversity of treatment concentrations and conditions. Not only is acute administration not comparable to long-term IL-1β production (as one would expect to see in vivo in chronic pain), but even when considering only acute administration in vitro, the modes of delivery and dosages in each study vary considerably. The lack of consistency on this matter makes the comparison of different experiments and preparations very difficult. The concentrations of IL-1β in vivo in an organism's normal resting state are known to be minuscule (Sommer, Sorkin, & Kress, 2006), yet there is no consensus on IL-1β concentrations following nerve injury or inflammation. Reported values vary depending on mode of delivery (e.g. i.t. injection, cannula perfusion) or stimulus by which IL-1β is produced (e.g. LPS, CFA, gp120), and they are also dependent on the location at which IL-1β levels are measured (e.g. serum, CSF). Thus, it was difficult to come up with treatment conditions in DMOTC that would definitively reproduce the injured or inflamed environment in the dorsal horn.

In summary, extrapolating findings from other studies in an effort to derive "high" and "low" concentrations with which to treat dorsal horn neurons in DMOTC and determining what effects should be expected with 32pM and 118pM under culture conditions is very difficult, as the preparations and dosages vary considerably within each study, and there appears to be a broad range of effective treatment concentrations depending on which neuronal or synaptic effect is being studied. Future research using DMOTC should be focused on establishing dose-response curves with IL-1β in dorsal horn neurons, and considering the ELISA results in the current project, studies of the time course of IL-1β action and peak concentrations in this preparation would also be very beneficial.

6.3 Possible Cellular Mechanisms of IL-1β Action

There is evidence that many cell types can express and release IL-1β in response to neuronal injury; these different cells can produce a wide range of pro-inflammatory and immunoregulatory mediators that can promote cell survival or contribute to cell death (Allan et al., 2005) (Figure 6-1). The classical IL-1β-mediated signaling pathways involve activation of MAPKs and NF-κB (specifically, p38MAPK in neurons and NF-κB in astrocytes [Srinivasan, Yen, Joseph, & Friedman, 2004]) and subsequent release of secondary pro-inflammatory mediators (e.g. PGE_2, nitric oxide, bradykinin), all of which have been shown to contribute to peripheral and central sensitization (Wang, Ehnert, Brenner, & Woolf, 2006; Allan et al., 2005).

In terms of known direct effects of IL-1β, there are a limited number of studies that provide evidence for the role of IL-1β as a neuromodulator. A recent study using primary cultures of rat hippocampal neurons found that IL-1β, but not other pro-inflammatory cytokines, effected two significant changes in neurons: IL-1β decreased the surface expression and Ser831 phosphorylation of the AMPA receptor subunit GluR1, and IL-1β also regulated NMDA-mediated Ca^{2+} influx (Lai, Swayze, El-Husseini, & Song, 2006). These effects were abolished when IL-1β blocking agents were employed. Specifically, IL-1β has been shown to dose-dependently enhance NMDA receptor function through activation of tyrosine kinases and subsequent NR2A/B subunit phosphorylation (Viviani et al., 2003). The application of IL-1β results in an increase in NMDA-induced intracellular Ca^{2+} concentration and this may contribute to glutamate-mediated neurodegeneration (Wang & Salter, 1994; Viviani et al., 2003). Inhibition of NMDA receptor activity or depletion of extracellular Ca^{2+} blocked the effects of IL-1β on

GluR1 phosphorylation and surface expression, suggesting that IL-1β selectively regulates AMPA receptor phosphorylation and surface expression through extracellular Ca^{2+} and an unknown mechanism involving NMDA receptor activity (Lai et al., 2006). Although there is no consensus on which intracellular mechanisms are producing these changes, the stress-activated protein kinase p38 and the NMDA receptor-activated kinases, such as Src, have been implicated (Viviani et al., 2003; Takagi, Nozaki, Sugino, Hattori, & Hashimoto, 2000).

In a study conducted by Bernardino et al. (2005), it was found that at low doses (1000 pg/mL), pre-treatment with IL-1β potentiated AMPA-induced excitotoxicity in hippocampal neurons, while at high doses (10,000 pg/mL), pre-treatment with IL-1β resulted in neuroprotection against AMPA-induced excitotoxicity.

Studies reporting direct effects of IL-1β on AMPA and NMDA receptor function in hippocampal neurons used treatment concentrations ranging from 10 to 50 000 pg/mL (Lai et al., 2006; Viviani et al., 2003; Bernardino et al., 2005). It should be noted that although detectable effects were reported for concentrations as low as 10 pg/mL, the highest treatment concentrations (i.e. 50 000 pg/mL) were typically used in experiments as these 'elicited the strongest response', though they may not be representative of IL-1β concentrations in an injured or inflamed environment. In this vein, it may be that the reported direct neuromodulatory effects of IL-1β are pharmacologic effects that do not occur in vivo following nerve injury or inflammation. As discussed previously, more research is required to determine not only the specific, direct effects of IL-1β in various parts of the CNS and in the periphery, but also the probable concentrations in physiological, in vivo neuropathic pain conditions.

Neuropathic pain studies conducted in the dorsal horn of the spinal cord have shown that injury-induced stimuli can produce profound changes in cellular properties of these neurons. Specifically, peripheral nerve injury has been shown to deplete VGLUT1, which may lead to a decrease in glutamate levels, and thus, a decrease in synaptic efficacy (Hughes, Polgar, Shehab, & Todd, 2004). In terms of glutamate receptors, all four subunits of the AMPA receptor have been shown to exist in the dorsal horn of the spinal cord: GluR2 is almost universally present at AMPA-containing synapses, and following peripheral noxious stimulation, GluR1 subunits in the dorsal horn become rapidly phosphorylated, demonstrating that plastic changes do occur at the synaptic level in the dorsal horn (Nagy, Al-Ayyan, Andrew, Fukaya, Watanabe, & Todd, 2004).

In light of these studies and what has been reported to occur in the brain, it may be that IL-1β has some direct influence on AMPA receptor subunits that would alter neuronal transmission and increase hyperexcitability; however, the concentrations at which this may occur in vivo, and the mechanisms by which this may take place have yet to be clearly established. The results obtained with IL-1β are different from what has been seen in our lab following BDNF treatment in DMOTC, or following CCI in acute slices. The latter two projects have revealed that following injury or administration of BDNF, putative excitatory neurons of the substantia gelatinosa become more excitable, and putative inhibitory neurons are disinhibited, leading to an overall increase in synaptic excitability in this region. Although these specific changes were not seen following IL-1β treatment, it may be that any perturbation of synaptic transmission in the substantia gelatinosa (e.g. a change in AMPA receptor subunit phosphorylation) can lead to pain.

6.4 Future Research

6.4.1 Morphological Analysis

Examining the morphology of DMOTC neurons is a necessary step towards understanding the intricate circuitry and neuronal phenotypes of the superficial dorsal horn. Electrophysiological categorization is merely one aspect of possible neuronal characteristics, and by incorporating morphological analysis, it would become possible to better classify and understand DMOTC dorsal horn neurons. Further, it is possible to conduct immunohistochemical analyses on DMOTC slices, providing the invaluable distinction between excitatory and inhibitory neurons. Currently in the Smith lab, immunohistochemistry is being done using stains for GAD65; it is also possible to stain for calbindin, VGLUT, or other transporters such as VGAT, and these techniques are presently being developed to get a clear picture of what is occurring in both acute slices and DMOTC slices.

Some preliminary work with DMOTC has also been done in regards to morphology; the neurons recorded from in this project were filled with Biocytin (see Chapter 2) as were neurons from another project in the lab (Van B. Lu: application of BDNF to DMOTC neurons). Morphological analysis has resulted in the identification of three distinct morphological phenotypes: Dorsoventral neurons, Islet-central neurons, and Radial neurons. These are similar to morphological types seen in acute slices of rat substantia gelatinosa; however, the DMOTC slices flatten out considerably during the course of the culture, and this does influence the appearance of the neurons. Again, analyses are preliminary and higher sample sizes are required.

6.4.2 Dose-Response Studies and IL-1RA

As has been previously suggested in this chapter, dose-response studies for IL-1β in DMOTC are critical at this stage of research. Employing the natural receptor antagonist IL-1RA in these studies would be an additional safeguard against spurious results, and in conjunction with ELISA analysis, would provide essential information on the effective concentrations and functions of IL-1β. As much of the research surrounding IL-1β in the context of neuropathic pain is behavioural and acute, there is a dire need for these long-term studies. Particularly since the results of this project were confounded by speculative treatment concentrations and unclear effective concentrations of IL-1β in DMOTC, dose-response experiments would provide a much-needed rationale for effects observed in this and other studies of neuropathic pain.

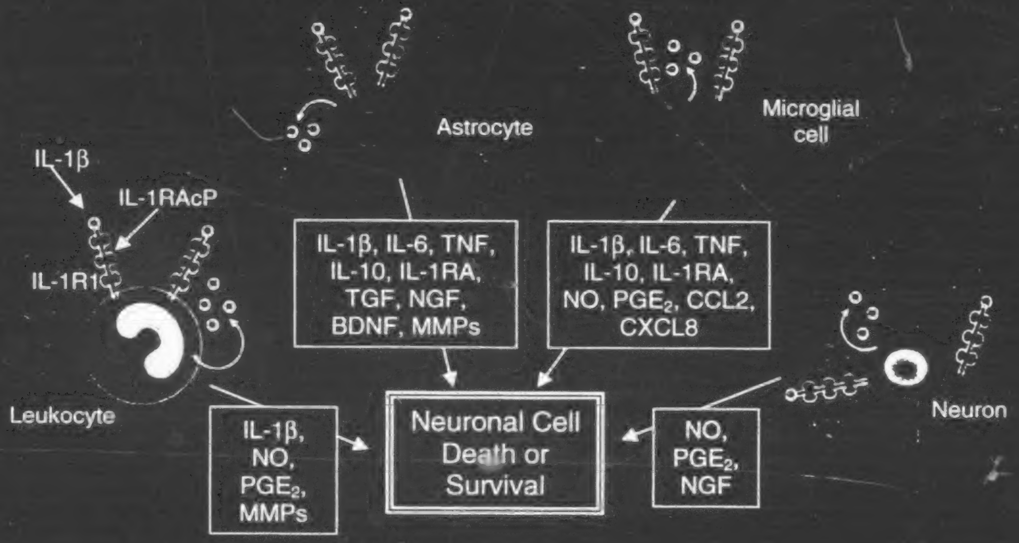
Astrocyte
Microglial cell
IL-1β
IL-1RAcP
IL-1R1
Leukocyte
IL-1β, IL-6, TNF, IL-10, IL-1RA, TGF, NGF, BDNF, MMPs
IL-1β, IL-6, TNF, IL-10, IL-1RA, NO, PGE₂, CCL2, CXCL8
Neuron
IL-1β, NO, PGE₂, MMPs
Neuronal Cell Death or Survival
NO, PGE₂, NGF

Figure 6-1. Summary of proposed cellular actions of IL-1β in neuronal injury.

6.5 References

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